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RELATION OF BARBERRY TO THE ORIGIN AND PERSISTENCE OF PHYSIOLOGIC FORMS OF PUCCINIA GRAMINIS[1]

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INTRODUCTION

It is now well known (*4, 5, 6, 9, 13*)[2] that new physiologic forms of *Puccinia graminis* Pers. may arise as the result of hybridization between existing forms or varieties on barberry. The evidence at present available indicates that hybridization probably accounts principally for the origin of forms, although Stakman, Levine, and Cotter (*9*) have shown that mutation in parasitism may also occur, though probably rarely. There is little information, however, regarding the origin of new forms through hybridization on barberries in nature. For this reason the writers made studies during the past several years to ascertain whether new physiologic forms could be obtained from aecial material and from uredial material near barberries. Preliminary statements of the results have been published (*7, 12*). These and other data have been combined with the results of earlier surveys and are presented in the following pages. Data are given also on the results of "selfing" pycnia resulting from inoculations with telial collections of unknown identity.

The writers were particularly interested in the varieties of *Puccinia graminis* that attack the common small grains; hence inoculations were made on barley (*Hordeum vulgare* L.) and oats (*Avena sativa* L.), or on wheat (*Triticum vulgare* Vill.),[3] oats, and rye (*Secale cereale* L.), to obtain preliminary indications of the identity of the variety of rust in question. Barley is susceptible to both the *tritici* and the *secalis* varieties; hence, it sometimes was used in the preliminary inoculations to "screen out" the *poae* and *agrostidis* varieties, neither of which develops well on it. The determination of physiologic forms within rust varieties was made by revised keys originally developed by Stakman and Levine (*8*) for physiologic forms of *P. graminis tritici* Eriks. and Henn.; by Bailey (*1*) for physiologic forms of *P. graminis avenae* Eriks. and Henn., and by Cotter and Levine (*3*) for physiologic forms of *P. graminis secalis* Eriks. and Henn.

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[2] Reference is made by number (italic) to Literature Cited, p. 968.

[3] According to the rules of botanical nomenclature the name of this species is *Triticum aestivum;* but as *T. vulgare* is in general use among agronomists and cereal pathologists and geneticists, the writers give preference to that form.

The relatively low percentage of infection resulting from inoculating with aeciospores may be due to several causes. *Puccinia graminis poae* Eriks. and Henn. and *P. graminis agrostidis* Eriks. and Henn.[4] are very prevalent on barberries in certain regions, and many of the collections probably were of these varieties. Then, too, most of the material was sent through the mails and much of it was not in good condition when received. Further, the germination of aeciospores is somewhat more uncertain and considerably more capricious than that of urediospores; and aeciospores do not usually retain their viability more than 3 weeks, even when kept cool and fairly dry.

VARIETIES ISOLATED

VARIETIES FROM AECIAL COLLECTIONS

Of 675 aecial collections of *Puccinia graminis* tested during the past 13 years, only 281 caused infection on wheat, oats, rye, or barley (table 1). It seems likely, therefore, that many of the collections were of the *poae* or the *agrostidis* variety. Of the 281 collections that caused infection on the common small grains, 96, or 34.2 percent, were of the *tritici* variety; 179, or 63.7 percent, were of the *secalis* variety; and only 6, or 2.1 percent, were of the *avenae* variety.

TABLE 1.—*Isolation of Puccinia graminis varieties from rusted barberries in the field, 1920–32*

Year	Collections tested	Total cultures identified	Isolations of *Puccinia graminis* var.—					
			Tritici		*Secalis*		*Avenae*	
	Number	*Number*	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*
1920	8	4	3	75.0	1	25.0		
1921	14	6	5	83.3	1	16.7		
1922	9	3	1	33.3	2	66.7		
1923	6	2			2	100.0		
1924	12	6	1	16.7	5	83.3		
1925	9	5	3	60.0	2	40.0		
1926	19	12			11	91.7	1	8.3
1927	27	19	8	42.1	9	47.4	2	10.5
1928	93	60	23	38.3	35	58.4	2	3.3
1929	64	41	18	43.9	23	56.1		
1930	131	42	16	38.1	26	61.9		
1931	124	32	12	37.5	19	59.4	1	3.1
1932	159	49	6	12.2	43	87.8		
Total	675	281	96	34.2	179	63.7	6	2.1

It seems quite likely that the high percentage of collections of *Puccinia graminis secalis* is due to the fact (1) that quackgrass (*Agropyron repens* (L.) Beauv.) is susceptible to this variety, (2) that this grass is very prevalent in the Northern States, where barberries become rusted, and (3) that many of the remaining barberry bushes are along fence rows, in pastures, on the edges of wood lots, along streams, and in other uncultivated places, where quackgrass is likely to be abundant. Furthermore, *Hordeum jubatum* L. and other species of *Hordeum* are very susceptible to *P. graminis secalis*, as are also various species of *Elymus*. There is therefore opportunity for *P. graminis secalis* to develop abundantly. *P. graminis tritici* attacks not only wheat but also the same grasses that *P. graminis secalis* attacks, with the exception of *Agropyron repens*. As just explained, however, the great

[4] The varietal name as written by Eriksson and Henning is *agrostis*. In order to conform with the other varietal names, however, the writers prefer to use the genitive form, *agrostidis*, as originally used by Eriksson.

abundance and the advantageous habitat of the last-named grass give *P. graminis secalis* the advantage, despite the fact that the acreage of wheat attacked by *P. graminis tritici* is far greater than the acreage of rye attacked by *P. graminis secalis*. The relative paucity of collections of *P. graminis avenae* probably is due to the fact that *Dactylis glomerata* L., *Festuca* spp., *Alopecurus* spp., *Glyceria*, and the other grasses susceptible to this variety usually are not particularly abundant near barberry bushes (*11*).

VARIETIES FROM UREDIAL MATERIAL NEAR INFECTED BARBERRIES

In addition to determining the relative prevalence of the varieties of *Puccinia graminis* occurring in the aecial stage on barberry bushes in nature, the writers made a similar study of uredial material on grains and grasses in the immediate vicinity (within 100 yards) of infected bushes. This study includes only such rust as almost certainly resulted from infected bushes. The results are summarized in table 2.

TABLE 2.—*Isolations of Puccinia graminis varieties from uredial collections obtained in close proximity to infected barberries, 1919–32*

Year	Cultures identified	Isolations of *Puccinia graminis* var.—					
		Tritici		*Secalis*		*Avenae*	
	Number	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*
1919	3	2	66.7	1	33.3		
1920	3	1	33.3	2	66.7		
1921	4	2	50.0	2	50.0		
1922	9	3	33.3	6	66.7		
1923	3			1	33.3	2	66.7
1924	4			3	75.0	1	25.0
1925	5	2	40.0	3	60.0		
1926	7	2	28.6	4	57.1	1	14.3
1927	21	9	42.9	8	38.1	4	19.0
1928	27	16	59.3	5	18.5	6	22.2
1929	7	4	57.1	3	42.9		
1930	8	5	62.5	1	12.5	2	25.0
1931	19	12	63.2	2	10.5	5	26.3
1932	18	14	77.8	4	22.2		
Total	138	72	52.2	45	32.6	21	15.2

A total of 138 collections of *Puccinia graminis* were identified. Of these, 72, or 52.2 percent, were of the *tritici* variety; 45, or 32.6 percent, of the *secalis* variety; and 21, or 15.2 percent, of the *avenae* variety. The fact that the percentage of collections of the *tritici* variety was higher than that of the *secalis* variety, whereas the opposite was true of the aecial collections, is probably due largely to conscious selection. The writers were most interested in determining physiologic forms of the *tritici* variety and therefore made special effort to collect grasses and grains likely to be infected with it. Hence the percentages do not necessarily give as accurate an indication of the relative prevalence of the varieties in nature as those for the aecial collections.

It is clear that a high percentage of *Puccinia graminis* on barberries in the Northern States is of the *secalis* variety. This variety of rust probably is more closely dependent on barberry for persistence from season to season than are the *tritici* and *avenae* varieties. This is partly because very little rye is grown in the South, where the uredial stage of *Puccinia graminis* can survive the winter. It is evident that

there is appreciable development of stem rust of rye only in those regions where there are barberries. Stem rust of rye can be prevented, for practical purposes, by eradicating barberries. While it is perfectly clear that barberry eradication reduces the severity of rust attacks on wheat and oats also, investigations by the writers have shown that the uredial stage of these rusts often survives the winter in the far South and, under favorable conditions, urediospores may be blown northward, to cause infection on wheat and oats in the Northern States.

PHYSIOLOGIC FORMS ISOLATED

Attempt was made to identify the physiologic forms, both in aecial and uredial collections. In addition, barberries were inoculated in the greenhouse with collections of teliospores and the pycnia were "selfed", as described later. The primary object was to find out to what extent new physiologic forms were arising through hybridization in nature. It was desired also to ascertain to what extent barberries make possible the persistence of existing forms. Theoretically barberries should be of great importance in these respects, and, practically, the results show that they are.

PUCCINIA GRAMINIS TRITICI

Physiologic Forms from Aecial Collections

The identity of the physiologic forms represented in the 94 isolations of *Puccinia graminis tritici* was determined, 26 forms being

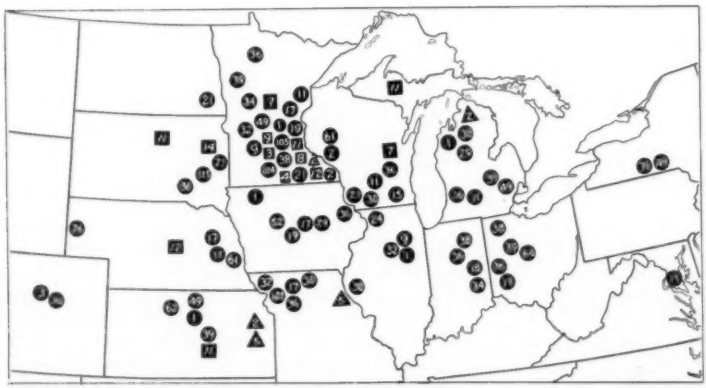

FIGURE 1.—Geographic distribution of physiologic forms (designated by number) of *Puccinia graminis*, isolated from naturally infected barberries. Circles indicate variety *tritici;* squares, *secalis;* triangles, *avenae*.

identified. Some of them were of frequent occurrence and wide distribution, others rare and limited; some were quite virulent, others fairly innocuous (table 3 and fig. 1; see also table 5).

It is worthy of note that so limited a number of isolations should have yielded so many different physiologic forms. This becomes especially striking when the number of physiologic forms isolated from field collections of infected grain plants is considered. In the latter case, 82 physiologic forms have been identified from more than 8,000

cultures made in the course of physiologic-form surveys; that is, on an average, not more than one form from each 100 uredial collections; whereas from aecial collections a different form was procured, on an average, from every fourth culture, although this ratio probably would not have persisted had more aecial collections been identified.

TABLE 3.—*Physiologic forms of Puccinia graminis tritici isolated from aecial collections, by States, 1920–32*

Form	Number of times form was found in—															Total
	Colorado	Illinois	Indiana	Iowa	Kansas	Maryland	Michigan	Minnesota	Missouri	Nebraska	New York	North Dakota	Ohio	South Dakota	Wisconsin	
1		1		1	1		1	2								6
2								1							1	2
3	1															1
9		1						1								2
11							1	1		1					1	4
15						1									1	2
17				1				1	1	1						4
18			1													1
19				1				1					1			3
21								2				1		1	1	5
24		1														1
29				1			1									2
32		1						1	1							3
34			1					1								2
36	1		1	2			1	8	2	2	1		1	2	3	24
38		1	2				1	2	2				1		2	11
39					2		1	1					1			5
49					1		1	3			1					6
52				1												1
61										1					1	2
62					1											1
66													1			1
102									1							1
104								1								1
105								2								2
125														1		1
Total	2	5	5	7	5	1	7	28	7	5	2	1	5	4	10	94
Percent	2.1	5.3	5.3	7.5	5.3	1.1	7.5	29.8	7.5	5.3	2.1	1.1	5.3	4.2	10.6	100
Number of forms	2	5	4	6	4	1	7	15	5	4	2	1	5	3	7	26

Form 36 was most common and most widely distributed. It was isolated 24 times in a total of 94 identifications, and it was found in 11 of 15 States in which collections were made. Next in order of occurrence and distribution was form 38, with a total of 11 isolations from 7 States. This was followed by a considerable drop in both occurrence and distribution was form 38, with a total of 11 isolations from 7 States. This was followed by a considerable drop in both occurrence and distribution, with 6 isolations each of forms 1 and 49 from 5 and 4 States, respectively. Forms 3, 18, 24, 52, 62, 66, 102, 104, and 125 were isolated only once each from the aecial material collected.

Forms 62, 102, 104, and 105 never have been isolated from any source other than rusted barberry. The original collections of forms 61 and 66 were made from naturally infected barberries, although subsequently they were obtained also from rusted grain in the field.

These facts indicate strongly that in addition to spreading much inoculum early in the season barberry bushes are extremely important in at least two respects: (1) They enable many physiologic forms to persist from season to season and (2) they make possible the origin of

new forms through hybridization. In 1919 it was pointed out by Stakman, Levine, and Leach (*10*) that the number of physiologic forms of *Puccinia graminis* seemed to be greater in those regions of the United States where there were many barberry bushes than in those where there were few. The results presented in this paper support this opinion and indicate clearly that barberry bushes should be eradicated in order to reduce the number of physiologic forms as well as to reduce the amount of inoculum, especially that of the early spring.

Of the forms isolated only from barberries and therefore probably produced on them during recent years, form 62 is the most virulent. It may cause heavy infection on all the differential hosts except Vernal and Khapli emmers. It is one of the most virulent of all forms, differing from form 15 only in its inability to infect Vernal normally

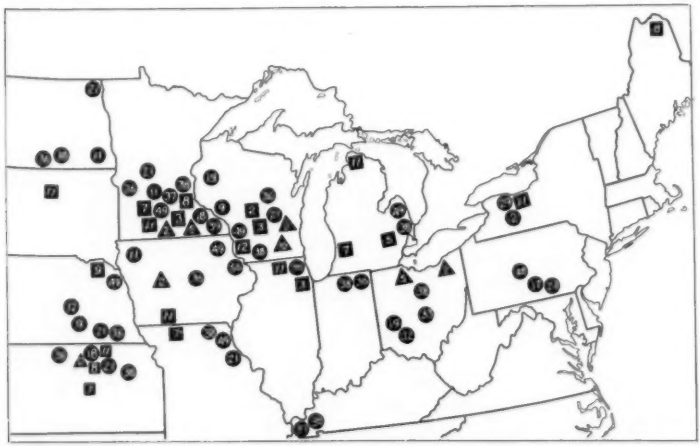

FIGURE 2.—Geographic distribution of physiologic forms (designated by number) of *Puccinia graminis*, collected in the vicinity of infected barberries. Circles indicate variety *tritici;* squares, *secalis;* triangles, *avenae.*

and in its tendency to produce type x infection on Marquis, Reliance, and Kota wheats; on these wheats form 15 produces type 4 infection (table 5). Under favorable conditions type x may develop sufficiently to cause heavy rust attack; hence form 62 must be considered potentially dangerous. The other forms isolated only from barberries are not particularly virulent, although it is worthy of note that on Vernal emmer both form 104 and 105 produce type x infection, which is a heavier infection than that caused by most other forms.

PHYSIOLOGIC FORMS FROM UREDIAL MATERIAL NEAR INFECTED BARBERRIES

The 71 collections of *Puccinia graminis tritici*, made on grains and grasses near infected barberries and completely identified, comprised 19 physiologic forms, a different form for approximately every four collections (table 4, fig. 2). These results support those obtained from inoculations with aeciospores. Here again it is clear that barberries are responsible for the persistence of many physiologic forms. When inoculations were made with urediospores collected at random,

in regions where barberries rust and in those where they do not, a different form appeared in 1 collection in 100, on an average. But when inoculations were made with urediospores collected near rusted barberries, a different form was obtained in 1 culture of every 4.

TABLE 4.—*Physiologic forms of Puccinia graminis tritici isolated from uredial collections obtained near infected barberries, by States, 1919–32*

Form	Number of times form was found in—														Total
	Illinois	Indiana	Iowa	Kansas	Michigan	Minnesota	Missouri	Nebraska	New York	North Dakota	Ohio	Pennsylvania	Virginia	Wisconsin	
2									1						1
9								1						1	2
11			1			1				1					3
12								1							1
17												1			1
18				2		2									4
19											1			1	2
21				1		3	1	1		1				2	9
23												1			1
29									1						1
32											1				1
33											1		1		2
36	1	1	1	2		4		1		4				2	16
37						1									1
38		2		1	1	1	2			1	1		1	4	14
39						1									1
48												1			1
49			2		1	2	1	1						2	9
56			1												1
Total	1	3	5	6	2	15	4	5	2	7	4	3	2	12	71
Percent	1.4	4.2	7.1	8.5	2.8	21.1	5.6	7.1	2.8	9.9	5.6	4.2	2.8	16.9	100
Number of forms	1	2	4	4	2	8	3	5	2	4	4	3	2	6	19

Forms 36 and 38 were the most prevalent, constituting 22.54 percent and 19.72 percent, respectively, of the total collections identified. Form 38 has been very prevalent in the soft red winter wheat area of the United States, where there still are many barberries; it is also very abundant in northern Mexico. Forms 21 and 49 were next in order of prevalence. By consulting table 5 it will be seen that these four forms could cause heavy rust on the principal types of wheat commonly grown in the wheat-growing regions of the northern half of the United States. Marquis and similar varieties are susceptible to three of these forms; the durums are resistant to forms 36 and 49, susceptible to form 21, and under favorable environmental conditions they are susceptible to form 38, which causes a type x+ infection. Reliance is immune from forms 21 and 49 but is susceptible to forms 36 and 38 (table 5). This variety reacts like Kanred, which often is immune in the field. It rusts heavily, however, when forms like 36 and 38 are present. Kota, which reacts like the widely grown variety, Ceres, is at least moderately susceptible to all four forms but seldom becomes heavily rusted in the field because of morphologic resistance.

Form 48 has never been found in the United States except near rusted barberries, although it has been reported several times from Canada, where it was first collected in 1929, on wheat. It is, however, of rare occurrence.

PUCCINIA GRAMINIS SECALIS

The physiologic forms of 27 aecial collections of *Puccinia graminis secalis* were identified, 9 forms being found. It seems significant that so many forms were isolated from so few collections. On an average, a different form was isolated from every third collection. Forms 7 and 11 were the most prevalent, constituting 25.93 and 29.63 percent, respectively, of the total (tables 6 and 8, and figs. 1 and 2).

TABLE 5.—*Types of infection*[a] *on differential varieties of wheat caused by physiologic forms of Puccinia graminis tritici isolated from aecia and from uredia near infected barberries, 1919–32*

Form	Reaction of differential varieties											
	Jenkin	Marquis	Reliance	Kota	Arnautka	Mindum	Spelmar	Kubanka	Acme	Einkorn	Vernal	Khapli
1	4	4	0	3	1	1	1	3	3	3	0	1
2	4	2	2	2	1	1	1	1	3	3	1	0
3	4	4	4	3	1	1	1	1	3	3	1	0
9	4	4	0	3	4	4	4	4	3	3	4	1
11	4	4	3	3	4	4	4	3	3	3	1	1
12	4	4	4	3	4	1	1	1	3	3	1	0
15	4	4	4	3	4	4	4	3	3	3	4	1
17	4	4	0	3	4	4	4	3	3	3	1	1
18	4	4	4	3	1	1	1	3	3	3	1	1
19	4	2	0	3	4	4	4	3	3	3	0	1
21	4	4	0	3	4	4	4	4	3	1	0	1
23	4	2	1	1	1	1	1	3	3	3	0	0
24	4	4	0	2	4	4	4	3	3	3	1	0
29	4	4	0	3	x	x	x	x	x	3	1	1
32	4	4	4	3	x	x	x	x	x	3	1	1
33	4	2	4	1	1	1	1	4	3	3	1	1
34	4	4	4	4	4	4	4	4	3	1	0	1
36	4	4	4	3	1	1	0	x	3	3	0	1
37	4	4	0	3	4	4	4	x	3	3	1	1
38	4	2	4	3	x	x	x	x	x	4	1	1
39	4	2	4	3	4	3	4	4	3	4	1	1
48	4	1	0	1	x	x	x	x	4	4	1	1
49	4	4	0	4	1	1	0	x	3	1	0	1
51	4	2	3	0	0	0	0	4	3	3	4	0
52	4	4	4	4	1	1	1	x	4	4	4	1
56	4	3	3	3	1	1	1	3	3	1	1	1
61[b]	4	4	0	3	0	0	0	x	4	4	0	0
62[c]	4	x	x	x	4	4	4	4	4	3	0	1
66[b]	4	2	4	0	0	0	0	x	1	3	0	0
67[d]	4	4	4	4	1	2	2	x	x	3	4	1
96[d]	4	x	4	x	4	4	4	4	3	3	1	1
101	4	4	4	4	1	0	0	x	x	3	0	1
102[c]	4	0	1	0	0	0	1	1	0	3	0	1
104[c]	4	x	0	0	0	0	0	0	0	3	x	0
105[c]	4	x	0	3	0	0	0	x	3	3	x	1
125	4	4	4	4	0	0	0	x	4	1	0	1
127[d]	4	4	3	3	1	1	1	x	x	0	0	1

[a] 0 signifies immune; 1, very resistant; 2, moderately resistant; 3, moderately susceptible; 4, very susceptible; x, heterogeneous (uredia very variable, various types of infection appearing on the same leaf, not due to mechanical mixture.

[b] First isolated from barberries.

[c] Isolated only from barberries.

[d] From selfing only.

TABLE 6.—*Physiologic forms of Puccinia graminis secalis and P. graminis avenae, isolated from aecial collections, by States, 1920–32*

NUMBER OF TIMES FORM WAS FOUND

Variety and form	Kansas	Maine	Michigan	Minnesota	Missouri	Nebraska	South Dakota	Wisconsin	Total
P. graminis secalis:									
3				1					1
4				1					1
5		1							1
7				6				1	7
8				1					1
9				3					3
11	3		2	2			1		8
12				3		1			4
14							1		1
Total	3	1	2	17		1	2	1	27
P. graminis avenae:									
2	1		1	2					4
5	1				1				2
Total	2		1	2	1				6

NUMBER OF FORMS

P. graminis secalis	1	1	1	7		1	2	1	9
P. graminis avenae	2		1	1	1				2

Form 5 is the only form that had not been isolated previously from uredial material. As it was subsequently obtained also from uredia near barberries, it probably had been formed recently.

From 28 uredial collections of *Puccinia graminis secalis* near infected barberries, 8 physiologic forms were isolated, again a large number of forms in proportion to the number of collections. Forms 7 and 11 were again the most prevalent (table 7).

PUCCINIA GRAMINIS AVENAE

Only six aecial collections of *Puccinia graminis avenae* were identified. Forms 2 and 5 occurred in the ratio of 2 to 1. Both are extremely common and widely distributed in the United States; consequently no particular significance attaches to their occurrence on barberries. However, an entirely new form, designated form 10, was obtained from oats near rusted barberries in Wisconsin. A special note on this form has been published by Cotter (*2*); hence details will not be repeated here, beyond calling attention to the fact that it may reasonably be concluded to have resulted from hybridization on barberries in nature. It is important to note that form 10 is much more virulent on the Richland group of oat varieties than either form 2 or form 5 (table 8).

TABLE 7.—*Physiologic forms of Puccinia graminis secalis and P. graminis avenae isolated from uredial collections made near infected barberries, by States, 1919–32*

NUMBER OF TIMES FORM WAS FOUND

Variety and form	Illinois	Iowa	Kansas	Maine	Michigan	Minnesota	Missouri	Nebraska	New York	Ohio	South Dakota	Wisconsin	Total
P. graminis secalis:													
2						1						1	1
3	2											1	4
5					1								1
7			3		1	3	1						8
8			2	1		1							4
9								1					1
11	1	1	1		2	1			1		1		8
12												1	1
Total	3	1	6	1	4	6	1	1	1		1	3	28
P. graminis avenae:													
2		1	1			1				1		1	5
5						2				1			3
10												2	2
Total		1	1			3				2		3	10

NUMBER OF FORMS

P. graminis secalis	2	1	3	1	3	4	1	1	1		1	3	8
P. graminis avenae		1	1			2				2		2	3

TABLE 8.—*Types of infection produced on differential varieties of rye and oats caused by physiologic forms of Puccinia graminis secalis and P. graminis avenae, respectively, isolated from aecia and from uredia near infected barberries, 1920–32*

Form	Varieties of rye and relative susceptibility to *P. graminis secalis*					Varieties of oats and mean infection type		
	Rosen	Swedish	Prolific	Dakold	Colorless	Minrus	Richland	Joanette
2	88.1	15.8	40.0			2	1	4
3	88.5	83.4	81.4	60.9	88.3			
4	93.3	81.8	62.6	61.3	96.9			
5	84.3	6.9	85.7			2	1	x
7	89.3	62.7	60.4	38.0	88.8			
8	67.8	41.4	62.1	13.6	79.4			
9	87.3	52.4	81.2	19.6	86.7			
10						2	4	x
11	87.4	46.7	58.7	17.4	86.6			
12	86.1	63.5	81.2	36.7	87.8			
14	85.8	39.9	53.9	10.8	68.3			

PHYSIOLOGIC FORMS FROM AECIA RESULTING FROM SELFED PYCNIA

To obtain still further information on the role of barberries in producing new physiologic forms and in perpetuating old ones, a number of "selfings" were made. The method was to inoculate barberries with collections of telial material, then transfer pycnial nectar from certain pycnia to others of the same collections, and inoculate differential hosts with the progeny of spores resulting from inoculations with spores from single aecia. There is, of course, no way of knowing certainly whether forms isolated were already present in the telial

stage, whether they resulted from segregation, or whether new combinations were made. The essential fact brought out by the results, however, is that in proportion to the number of cultures isolated a large number of forms was always obtained. From some of the aecia resulting from inoculation with teliospores, 2 forms, and in one case, 4 forms, were obtained. The average ratio between the number of forms isolated and the number of telial collections used for inoculation was 3 to 2. The ratio was approximately the same with *Puccinia graminis tritici* and *P. graminis secalis*, being 1.62 to 1 and 1.56 to 1, respectively. As has been previously pointed out, when determinations of physiologic forms of *P. graminis tritici* were made from uredial material collected at random, one physiologic form was isolated from approximately every 100 collections. From aecial material resulting from inoculating barberries with teliospores in the greenhouse, however, the average number of forms isolated from each collection was 0.39. This would seem to indicate, again, that the

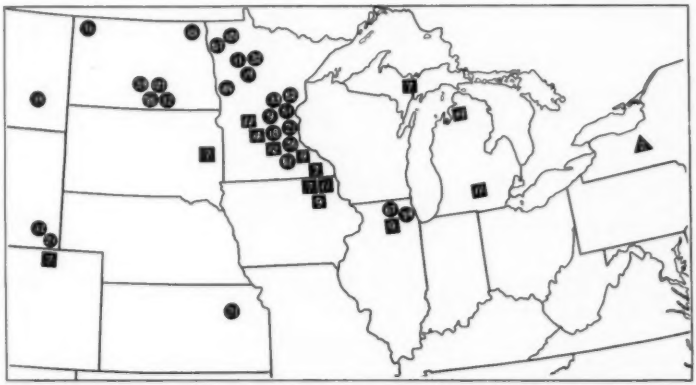

FIGURE 3.—Geographic distribution of physiologic forms (designated by number) of *Puccinia graminis*, segregated after passing through susceptible barberry. Circles indicate variety *tritici;* squares, *secalis;* triangle, *avenae*.

eradication of barberries will result in a diminution of the number of forms.

From a total of 30 cultures of *Puccinia graminis tritici*, 17 different physiologic forms were isolated (table 10). Those obtained most commonly were forms 67 and 101, not forms 36 and 38, which were most frequently isolated from aecial collections and from uredial collections made near barberries in nature. Nevertheless, form 36 follows forms 67 and 101 in order of frequency and is very similar to them in pathogenicity, but form 38 was not obtained at all. The complete list of forms isolated is given in table 10. (See also fig. 3.)

Forms 67, 96, and 127, which were isolated in these experiments, have never been obtained from uredial material. Of these, forms 67 and 96 had previously been produced as a result of artificial hybridization and form 127 was first isolated as a result of selfing but has been produced subsequently by hybridization also. Form 101 had been identified from uredial material collected in Bulgaria but has not been found in nature in North America. Its only recorded appearance on this continent is on barberries on which pycnia were selfed.

Forms 7, 9, 11, and 12 of *Puccinia graminis secalis* also were isolated from aecia produced on barberries in nature and from uredia obtained near barberry bushes. Of these, 7 and 11 have been most prevalent throughout. Forms 4 and 14 were obtained once each from the aecial material and from the products of selfing, but form 14 has never been obtained from uredial isolations.

It seems probable from the results given above that still other new forms might be produced if there were opportunity for certain telial material to cause infection on barberries in nature.

TABLE 9.—*Physiologic forms of Puccinia graminis varieties isolated from aecia produced as a result of selfing pycnia on barberries inoculated with collections of teliospores, 1926–32*

Year	Number of selfings [a] and number of forms isolated of *P. graminis*							
	Total		Var. *tritici*		Var. *secalis*		Var. *avenae*	
	Selfings	Forms isolated	Selfings	Forms isolated	Selfings	Forms isolated	Selfings	Forms isolated
1926	1	4	1	4				
1927	2	5	1	2	1	3		
1928	1	1	1	1				
1929	5	7	2	2	3	5		
1930	2	3	1	1	1	2		
1931	6	7	4	5	2	2		
1932	14	21	11	19	2	2	1	0
Total	31	48	21	34	9	14	1	0
Ratio of number of forms to selfings	1.55:1		1.62:1		1.56:1		0:1	

[a] This indicates the number of times barberries were inoculated with teliospore collections. Several pycnia were selfed in each case and inoculations made with aeciospores from the resulting aecia. Urediospores were then used for inoculating differential varieties.

DISCUSSION AND CONCLUSIONS

The results presented in the foregoing pages show clearly that barberries are important in two ways besides that of producing abundant inoculum early in the spring: (1) They enable new physiologic forms to arise through hybridization, and (2) they apparently enable many forms to persist and multiply.

The results of these field studies support the opinion, based on previous greenhouse experiments, that new forms arise on barberries in nature. Four forms of *Puccinia graminis tritici* have been isolated only from naturally infected barberries in the field, and two others were isolated from barberries before they were found elsewhere. Form 5 of *P. graminis secalis* has been isolated only from aecia on barberries and from uredia in close proximity to infected barberries, while form 10 of *P. graminis avenae* has been found only near infected barberries. The writers are convinced that these forms were of recent origin, indicating that new forms are still arising on susceptible barberries. The significance of the results is increased by the fact that these forms had not been found previously in extensive physiologic-form surveys made each year since 1917.

There is strong circumstantial evidence that the numerous physiologic forms now in existence have arisen principally as a result of hybridization. Despite very extensive experiments with physiologic

TABLE 10.—*Physiologic forms of Puccinia graminis varieties isolated from aecia produced as a result of selfing pycnia on barberries inoculated with collections of teliospores, by States, 1926–32*

NUMBER OF TIMES FORM WAS FOUND

Variety and form	Colorado	Illinois	Iowa	Kansas	Michigan	Minnesota	Montana	New York	North Dakota	South Dakota	Wyoming	Total
P. graminis tritici:												
9						1						1
11						1						1
18						1						1
19						1						1
21				1		1					1	3
32						1					1	2
33						1						1
34		1							1			2
36						1	1		1			3
49						1						1
51						1						1
52						1			1			2
61						1						1
67		1				3						4
96									1			1
101						3			1			4
127									1			1
Total		2		1		18	1		6		2	30
P. graminis secalis:												
4						1						1
7	1		1		2	4				1		9
9		1	1			1						3
11			1		1	12						14
12						1						1
14					1							1
Total	1	1	3		4	19				1		29
P. graminis avenae:												
2								1				1

NUMBER OF FORMS

P. graminis tritici		2		1		14	1		6		2	17
P. graminis secalis	1	1	3		3	5				1		6
P. graminis avenae								1				1

forms since 1916, the writers have observed only two definite cases of mutation in parasitism, which are believed to be the only ones on record for *Puccinia graminis*. Color mutations are not infrequent, but mutation in pathogenicity seems rare. On the other hand, there is abundant evidence that new forms arise frequently through hybridization. It seems reasonable, therefore, to assume that most physiologic forms have arisen in this way.

The large number of forms in regions where barberries become heavily rusted and the smaller number in regions where the aecial stage is rare also support the hypotheses just stated. As already pointed out by Waterhouse(*13*), the presence of a large number of forms in the Mississippi Valley of North America and of a small number in Australia seems significant. Furthermore, in the rather isolated "Inland Empire" of the Pacific Northwest of the United States, where barberries rarely become rusted, there seem to be relatively few forms of *Puccinia graminis tritici*, although wheat has long been grown extensively in the region and club wheats, which are sus-

ceptible to nearly all forms known in North America, have been grown commonly.

It is a striking fact, also, that so large a number of forms can be isolated from aecia and from uredia on grains and grasses in close proximity to barberry bushes. For example, a different form of *Puccinia graminis tritici* was isolated from approximately every third collection of aecial material, whereas a different form was isolated from only each 100 collections of uredial material collected at random (tables 11 and 12).

TABLE 11.—*Summary of the identity and number of physiologic forms of Puccinia graminis varieties isolated from aecial and telial collections, and uredial collections made near barberries, 1920–32*

Form	Number of times each form was found							
	Aecial collections from infected barberries			Uredial collections near infected barberries			Telial collections selfed on barberries	
	Var. *tritici*	Var. *secalis*	Var. *avenae*	Var. *tritici*	Var. *secalis*	Var. *avenae*	Var. *tritici*	Var. *secalis*
1	6							
2	2		4	1	1	5		
3	1	1			4			
4		1						1
5		1	2		1	3		
7		7			8			9
8		1			4			
9	2	3		2	1		1	3
10 [a]						2		
11	4	8		3	8		1	14
12		4		1	1			1
14		1						1
15	2							
17	4			1				
18	1			4			1	
19	3			2			1	
21	5			9			3	
23				1				
24	1							
29	2			1				
32	3			1			2	
33				2			1	
34	2						2	
36	24			16			3	
37				1				
38	11			14				
39	5			1				
48				1				
49	6			9			1	
51							1	
52	1						2	
56				1				
61 [b]	2						1	
62 [c]	1							
66 [b]	1							
67 [d]							4	
96 [d]							1	
101							4	
102 [c]	1							
104 [c]	1							
105 [c]	2							
125	1							
127 [d]							1	
Total	94	27	6	71	28	10	30	29
Number of forms	26	9	2	19	8	3	17	6

[a] Found near infected barberries only.
[b] First isolated from barberries.
[c] Isolated only from barberries.
[d] From selfing only.

TABLE 12.—*Physiologic forms isolated as compared with collections of Puccinia graminis varieties cultured*

Variety	Physiologic forms isolated from collections of designated spore stages							
	Aecial		Uredial		Telial		Weighted average [a]	
	Ratio [b]	Percent [c]	Ratio [b]	Percent [c]	Ratio [b]	Percent [c]	Ratio [b]	Percent [c]
P. graminis tritici	3.62:1	27.66	3.74:1	26.76	1.76:1	56.67	3.15:1	31.72
P. graminis secalis	3.00:1	33.33	3.50:1	28.57	4.83:1	20.68	3.65:1	27.38
P. graminis avenae	3.00:1	33.33	3.33:1	30.00			3.20:1	31.25
Weighted average for spore stages	3.43:1	29.13	3.63:1	27.52	2.57:1	38.98	3.28:1	30.51

[a] As a basis of comparison, the ratio for *P. graminis tritici* was approximately 100:1 in the general physiologic-form survey, when collections were made at random in the United States and Mexico, that is, from approximately each 100 collections a different form was obtained.
[b] Ratio of number of collections cultured to number of physiologic forms found.
[c] Percentage of physiologic forms in terms of the number of collections cultured.

Evidently barberries are responsible, therefore, not only for the production of new forms, but also to a considerable extent for their persistence; and in previous publications abundant evidence has been presented showing that barberries also are responsible for the dissemination of a tremendous amount of inoculum early in the growing season.

SUMMARY

The writers have investigated the role of barberries in the production and perpetuation of physiologic forms of *Puccinia graminis* in nature.

During the past 13 years inoculations were made on the common small grains with material from 675 aecial collections of *Puccinia graminis* obtained from the northern part of the United States. Of these, 281 caused infection, 34.2 percent being of the *tritici* variety, 63.7 percent of the *secalis* variety, and only 2.1 percent of the *avenae* variety.

The relative prevalence of the different varieties of *P. graminis* probably is governed to a considerable extent by the distribution of wild grasses susceptible to the different varieties.

The varietal identity of 138 uredial collections of *P. graminis* obtained within 100 yards of rusted barberries also was determined, with the following percentages: *tritici*, 52.2 percent; *secalis*, 32.6 percent; and *avenae*, 15.2 percent. These percentages probably were affected by a certain amount of conscious selection of hosts known to be susceptible to certain rust varieties.

The results given above, supplemented by other observations, indicate that stem rust of rye (*P. graminis secalis*) is almost wholly dependent on barberries for its persistence in the United States.

From 94 aecial collections of *P. graminis tritici*, 26 physiologic forms were isolated, a different form from approximately every 4 collections, whereas from about 8,000 uredial collections made at random over a period of years a different form was isolated from about every 100 collections.

Of the physiologic forms isolated from *P. graminis tritici*, forms 36 and 38 were the most prevalent.

Four forms (62, 102, 104, and 105) never have been isolated from any source other than rusted barberries. Forms 61 and 66 were isolated first from rusted barberries but subsequently were isolated from rusted wheat also.

From 71 uredial collections of *P. graminis tritici* made near rusted barberries, 19 physiologic forms were obtained, one of which (form 48) never has been found elsewhere in the United States, although reported several times from Canada.

The results indicate that barberries in nature are responsible for the production of new physiologic forms of *P. graminis tritici* as well as for the persistence of numerous forms.

Twenty-seven aecial collections of *P. graminis secalis* comprised 9 physiologic forms, and 28 uredial collections obtained near rusted bushes comprised 8 forms, a far larger number in proportion to the number of collections than in the case of uredial collections made at random. Forms 7 and 11 were the most prevalent; form 5 has been obtained only from aecia or uredia formed near infected barberries, and form 14 was isolated only from aecial collections or was the product of "selfing" on barberry.

Only six aecial collections of *P. graminis avenae* were identified, forms 2 and 5, which are widely distributed in the United States, being isolated. A new form (10), however, was isolated from oats near rusted barberries. It is far more virulent on the Richland group of oat varieties than either of the other two forms mentioned.

When barberries were inoculated with telial material in the greenhouse, the ratio between the number of forms isolated and the number of telial collections used for inoculating was 2 to 5. For example, 17 forms were isolated from 30 cultures of *P. graminis tritici* and 6 forms from 29 cultures of *P. graminis secalis*.

Forms 67, 96, and 127 of *P. graminis tritici*, isolated as a result of inoculating barberries with teliospores, never have been obtained from uredial material in the field, and another form (101) has never been found in the United States except on artificially inoculated barberries, although it was isolated from uredial material collected in Bulgaria.

Two physiologic forms (4 and 14) of *P. graminis secalis* were isolated from artificially inoculated barberries and from naturally infected bushes in the field, but form 14 has never been isolated from uredial material.

It is concluded that in nature barberries are important in the production and persistence of physiologic forms and in the persistence of certain varieties of rust, especially *P. graminis secalis* and probably *P. graminis agrostidis* and *P. graminis poae* also.

LITERATURE CITED

(1) Bailey, D. L.
1925. Physiologic specialization in Puccinia graminis avenae Erikss. and Henn. Minn. Agr. Expt. Sta. Tech. Bull. 35, 33 pp., illus.

(2) Cotter, R. U.
1932. A new form of oat stem rust from a barberry area. Phytopathology 22: 788–789.

(3) COTTER, R. U., and LEVINE, M. N.
1932. PHYSIOLOGIC SPECIALIZATION IN PUCCINIA GRAMINIS SECALIS. Jour. Agr. Research 45: 297–315, illus.
(4) LEVINE, M. N., and COTTER, R. U.
1931. A SYNTHETIC PRODUCTION OF PUCCINIA GRAMINIS HORDEI F. AND J. (Abstract) Phytopathology 21: 107.
(5) NEWTON, M., and JOHNSON, T.
1932. SPECIALIZATION AND HYBRIDIZATION OF WHEAT STEM RUST, PUCCINIA GRAMINIS TRITICI, IN CANADA. Canada Dept. Agr. Bull. 160, 60 pp., illus.
(6) ——— JOHNSON, T., and BROWN, A. M.
1930. A PRELIMINARY STUDY ON THE HYBRIDIZATION OF PHYSIOLOGIC FORMS OF PUCCINIA GRAMINIS TRITICI. Sci. Agr. 10: 721–731, illus.
(7) STAKMAN, E. C., HINES, L., COTTER, R. U., and LEVINE, M. N.
1932. PHYSIOLOGIC FORMS OF PUCCINIA GRAMINIS PRODUCED ON BARBERRIES IN NATURE. (Abstract) Phytopathology 22: 25.
(8) ——— and LEVINE, M. N.
1922. THE DETERMINATION OF BIOLOGIC FORMS OF PUCCINIA GRAMINIS ON TRITICUM SPP. Minn. Agr. Expt. Sta. Tech. Bull. 8, 10 pp., illus.
(9) ——— LEVINE, M. N., and COTTER, R. U.
1930. ORIGIN OF PHYSIOLOGIC FORMS OF PUCCINIA GRAMINIS THROUGH HYBRIDIZATION AND MUTATION. Sci. Agr. 10: 707–720.
(10) ——— LEVINE, M. N., and LEACH, J. G.
1919. NEW BIOLOGIC FORMS OF PUCCINIA GRAMINIS. Jour. Agr. Research 16: 103–105.
(11) ——— and PIEMEISEL, F. J.
1917. BIOLOGI CF ORMSOF PUCCINIA GRAMINIS ON CEREALS AND GRASSES. Jour. Agr. Research 10: 429–496, illus.
(12) WALLACE, J. M.
1932. PHYSIOLOGIC SPECIALIZATION AS A FACTOR IN THE EPIPHYTOLOGY OF PUCCINIA GRAMINIS TRITICI. Phytopathology 22: 105–142, illus.
(13) WATERHOUSE, W. L.
1929. A PRELIMINARY ACCOUNT OF THE ORIGIN OF TWO NEW AUSTRALIAN PHYSIOLOGIC FORMS OF PUCCINIA GRAMINIS TRITICI. Linn. Soc. N. S. Wales, Proc. 54: [96]–106, illus.

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SIZE AND ARRANGEMENT OF PLOTS FOR YIELD TESTS WITH CULTIVATED MUSHROOMS [1]

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INTRODUCTION

Numerous studies have been made to determine the most efficient plot technic for various agronomic and horticultural crops under different conditions. A bibliography on this subject has been published recently.[3]

In the few papers dealing with the results of yield tests in experiments with the cultivated mushroom (*Agaricus campestris* L.) a discussion of plot technic has been entirely neglected. Yet plot technic is of unusual importance in mushroom tests since there is normally more variability in mushroom beds than in ordinary field plots and the experimenter has an unusual opportunity to control conditions. Furthermore, the number and size of experimental plots must be reduced to a minimum because the labor and expense of yield tests is increased tremendously by the mushroom's habit of fruiting continuously for 3 or 4 months.

In commercial practice mushrooms are usually grown on shelf beds in windowless sheds or houses. An average mushroom house is about 60 feet long and contains 2 tiers of 5 or 6 shelf beds in which the individual beds are 24 inches apart. The beds are 5 or 6 feet wide and run the entire length of the house, except for a narrow passageway at each end of the house.[4] As a result of the placing of uprights and bed supports at 4-foot intervals along the bed (fig. 1), the beds are normally subdivided into small sections 4 feet wide and extending across the bed.

These structural features suggest at least four convenient units of bed space which might be used for yield trials—entire houses, tiers of beds, beds, and sections.

Practical experience soon shows that entire houses are not suitable, for differences in compost heaps and in the prevalence of fungus diseases and pests in the various houses more than counterbalance the advantages gained by the large area harvested. Tiers of beds are preferable to entire houses, since comparisons may be made among test areas located in the same house, and since the beds are made from the same compost heap. For most experiments, however, this arrangement is not satisfactory because it precludes replication of the experimental areas owing to the fact that there are only two tiers of beds in a house. These objections do not apply to the use of beds

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[3] GARBER, R. J., LOVE, H. H., MOORES, C. A., and KIESSELBACH, T. A. STANDARDIZATION OF FIELD EXPERIMENTS. Jour. Amer. Soc. Agron. 22: 1056–1061. 1930.

[4] For a perspective drawing of a standard mushroom house, see the following publication: LAMBERT, E. B. MUSHROOM GROWING IN THE UNITED STATES. U.S. Dept. Agr. Circ. 251, 35 pp., illus. 1932.

or smaller plots, such as sections or groups of sections, for test units. Therefore, for most experiments beds or parts of beds would seem to be, a priori, the proper experimental units.

The choice between entire beds and smaller plots depends largely on the comparative variability of the yields from these units. The experiments outlined in this paper were designed primarily to throw light on this question and on the problem of plot arrangement.

MATERIAL AND METHODS

The experiments were conducted as simple uniformity trials. They were made in the Department's experimental mushroom cellar at the Arlington Experiment Farm, Rosslyn, Va., and in commercial houses

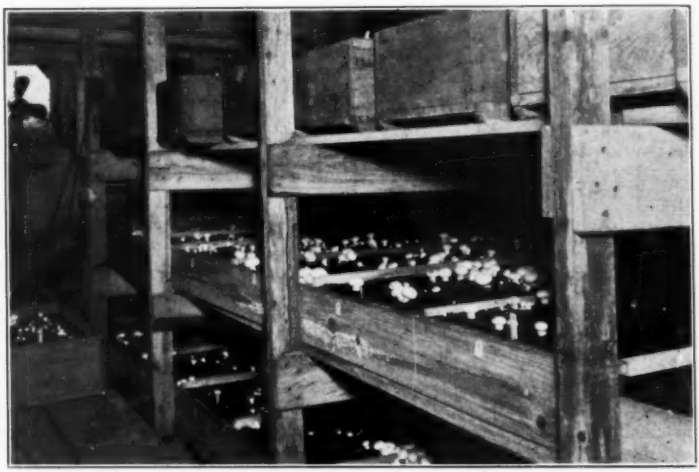

FIGURE 1.—Experimental mushroom beds at Arlington Experiment Farm, Rosslyn, Va., divided into ½-section plots 2 feet wide and extending across the bed. Note the placing of upright supports at 4-foot intervals; this is the standard arrangement in commercial houses and the beds as a matter of convenience are usually filled, with sections as unit areas, by unloading 10 or 12 bushels of compost at one time in the area between upright supports.

at Downingtown and Coatesville, Pa. The manure was composted in the regular manner, and the beds were filled with compost at the rate of approximately 1 bushel for 2 square feet of bed space. At Arlington Farm and Downingtown the beds were filled by emptying 10 bushel baskets into each 4-foot section on beds 5 feet wide and 12 bushel baskets into each section on beds 6 feet wide. At Coatesville 2 sections were filled at one time with 24 bushels of compost. In each experiment the composting in the heap was done as uniformly as possible, the same batch of spawn was used for the entire surface, and the casing soil was from a single source in each house. The sections at the ends of the beds were discarded because the yields from these sections are frequently reduced by excessive drafts. The picking was done at intervals of from 1 to 3 days until the beds were practically exhausted. In this connection it should be noted that the production period was terminated at Arlington Farm by a spell of hot weather

and the lower beds, which were slow to start, were injured more than the upper beds.

At Arlington Farm the total experimental area consisted of 5 beds each divided into 10 plots one half section (2 feet) wide (fig. 1). At Downingtown 5 beds were each divided into 10 plots one section (4 feet) wide. At Coatesville 4 beds were each divided into 10 plots one section (4 feet) wide. The yields given in table 1 for the separate plots are the sums of all the daily yields taken over a period of 3 months for each plot.

The "analysis of variance" method, devised and described by Fisher,[5] was used for analyzing the data. An arithmetical procedure was followed similar to that outlined by Fisher and Wishart.[6]

ANALYSIS OF YIELD DATA FROM UNIFORMITY TRIALS

ARLINGTON EXPERIMENT FARM, ROSSLYN, VA.

The yields of the 50 individual plots on the 5 experimental beds at Arlington Farm are given in table 1. If the 10 plots are considered as separate treatments and the beds as replicate blocks of these treatments, an analysis of variance, as shown in table 2, may be calculated that helps to clear up many of the points with which we are concerned.

TABLE 1.—*Yields of mushrooms in 3 uniformity trials*

YIELD PER 10-SQUARE-FOOT PLOT IN EXPERIMENTAL HOUSE AT ARLINGTON EXPERIMENT FARM, ROSSLYN, VA.

Bed no.	Yield from plot no.—									
	1	2	3	4	5	6	7	8	9	10
	Ounces	*Ounces*	*Ounces*	*Ounces*	*Ounces*	*Ounces*	*Ounces*	*Ounces*	*Ounces*	*Ounces*
1	382	263	242	312	184	226	283	273	272	262
2	286	338	239	336	239	297	216	231	220	266
3	153	154	143	175	167	203	209	170	203	201
4	145	143	158	170	176	174	127	167	133	153
5	162	148	158	194	176	181	183	239	210	193

YIELD PER 24-SQUARE-FOOT PLOT IN A COMMERCIAL MUSHROOM HOUSE AT DOWNINGTOWN, PA.

	Pounds	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*
1	21	21	25	24	28	30	20	24	25	24
2	36	27	32	27	28	27	24	38	30	23
3	20	20	20	29	15	24	36	29	21	30
4	28	25	16	22	23	24	30	21	20	27
5	24	21	19	36	26	33	28	24	32	23

YIELD PER 24-SQUARE-FOOT PLOT IN A COMMERCIAL MUSHROOM HOUSE AT COATESVILLE, PA.

1	31	36	42	37	50	45	42	40	43	37
2	42	41	41	42	41	31	45	47	45	42
3	43	40	41	40	40	50	37	45	46	39
4	31	34	42	35	42	37	41	41	41	43

[5] FISHER, R. A. STATISTICAL METHODS FOR RESEARCH WORKERS. Ed. 3, rev. and enl., 283 p., illus. Edinburgh and London. 1930.

[6] ——— and WISHART, J. THE ARRANGEMENT OF FIELD EXPERIMENTS AND THE STATISTICAL REDUCTION OF THE RESULTS. Imp. Bur. Soil Sci. Tech. Commun. no. 10, 24 pp. 1930.

TABLE 2.—*Analysis of variance of yields of mushrooms harvested in 3 locations from plots of different sizes, and arrangement*

ARLINGTON EXPERIMENT FARM, ROSSLYN, VA. (YIELD IN OUNCES)

Arrangement of plots corresponding to fig. 2	Plots			Blocks or beds (total area)	Variation	Degrees of freedom [a]	Sum of squares [b]	Variance [c]	z [d]	Standard deviation [e]	Coefficient of variability of—		Lowest significant difference (per cent between means of 5 replications) [h]	Efficiency (per cent on an area basis) [i]
	Width	Per block	Area of each (sections or parts of sections)								Single plot [f]	Mean of 5 replications [g]		
	Feet	*Number*		*Number*										
A	2	10	½	5	Between beds	4	115,731.6	28,932.90	1.5440	36.32	17.24	7.71	23.13	100
					Between columns	9	10,432.9	1,159.21						
					Interaction	36	47,486.0	1,319.06						
					Total	49	173,650.5							
B	4	5	1	5	Between beds	4	231,463.5	57,865.87	1.4316	57.47	13.6	6.08	18.24	80.3
					Within beds	20	66,058.5	3,302.92						
					Total	24	297,522.0							
C	6	3	1½	5	Between beds	4	322,002.0	85,504.4	1.3168	78.35	12.4	5.54	16.62	68.3
					Within beds	11	67,543.0	6,140						
					Total	15	389,545.0							
D	4	4	2 half	5	Between bed	4	165,479.3	41,369.82	1.6733	39.41	9.57	4.28	12.84	162.2
					Within beds	15	23,297.5	1,553.16						
					Total	19	188,776.8							

COMMERCIAL MUSHROOM HOUSE AT DOWNINGTOWN, PA. (YIELD IN POUNDS)

E	4	10	1	5	Between beds	4	261.6	65.40	0.47468	5.01	19.7	8.81	26.43	100
					Between columns	9	154.4	17.15						
					Interaction	36	906.0	25.17						
					Total	49	1,322.0							
F	8	5	2	5	Between beds	4	523.0	135.8	.47328	7.26	14.8	6.62	19.86	88
					Between columns	4	50.8	12.7						
					Interaction	16	864.0	52.7						
					Total	24	1,437.8							

COMMERCIAL MUSHROOM HOUSE AT COATESVILLE, PA. (YIELD IN POUNDS)

G	4	10	1	4	Between beds	3	74.2	24.9	----------	4.3	10.6	4.74	14.22	100
					Between columns	9	214.4	23.8						
					Interaction	27	500.8	18.5						
					Total	39	789.4							
H	8	5	2	4	Between beds	3	134	44.5	----------	5.9	7.2	3.22	9.66	108
					Between columns	4	281	70.2						
					Interaction	12	488	37.3						
					Total	19	903							
I	8	4	2	4	Between beds	3	100.7	33.90	----------	4.23	4.1	1.8	5.5	334
					Between columns	3	126.5	42.16						
					Interaction	9	160.8	17.87						
					Total	15	388.0							

[a] Number of independent comparisons.
[b] Sum of the squares of the deviations from the mean yield.
[c] Variance or mean square = $\frac{\text{sum of squares}}{\text{degrees of freedom}}$.
[d] z = one half of the difference between the natural logarithms of the mean squares between beds and of error.
[e] Standard error (standard deviation) = square root of variance.
[f] Coefficient of variability = standard error in percentage of the mean yield = $\frac{\text{S.E.} \times 100}{\text{mean yield}}$.
[g] Coefficient of variability divided by the square root of 5.
[h] $\frac{3 \times \text{CV}}{\sqrt{5}}$. (Odds approximately 20 to 1 against the occurrence due to chance of a difference as great or greater.)
[i] See p. 976.

In the reduction of the data the first objective was to determine the comparative efficiency of beds taken as a whole and of smaller plots such as sections or groups of sections. The data indicate higher variability between beds than between smaller plots (within beds) and lend themselves to the application of Fisher's z test to determine the statistical significance of this difference. When Fisher's table VI is consulted it is apparent that the observed value of z exceeds the 1-percent point in all cases, and it may be concluded that the differences are significant. The odds are more than 100 to 1 that such a difference would not occur as a result of chance variation. Since the variance between beds was significantly greater than the variance within beds, the data indicate that greater precision can be expected with smaller plots, and justify the arrangement of plots in the beds so that the variance between beds can be eliminated in calculating the standard error. In practice this would mean the random distribution of treatments on the beds with the restriction that each treatment shall occur once on each bed.

Conceivably, there might be conditions in the mushroom house that would bring about a consistently larger yield at one end of several of the beds analogous to the fertility gradients frequently encountered in soil-heterogeneity studies. Under these conditions there probably would be justification for arranging the plots in 5 by 5 Latin squares, in which the plots in each bed would constitute the rows and the plots one above the other or opposite one another in the house would constitute the columns of the square. Since all the plots have been treated uniformly the justification for such a procedure can be tested from the data in table 1 by calculating and comparing the variance between columns with the variance within columns and blocks. This comparison also is given in the analysis of variance shown in table 2. In this case the variance due to the interaction of beds and columns (error) is larger than the variance between columns, indicating that there would be no significant gain in the precision of the experiment by arranging the plots in a Latin square.

The next point of interest to be considered is the gain or loss in efficiency from the use of different-sized plots, such as one-half section (10 square feet), full section (20 square feet), or 1½ sections (30 square feet). In a series of analysis of variance as shown in table 2 the standard error in percentage of the mean, or coefficient of variability, was found to be reduced from 17.24 for one-half sections to 13.6 for entire sections, and 12.4 for 1½ sections (triple plots). On a plot basis this indicates a gain in precision with increase in plot size, but full-section plots take up twice as much space as the ½-section plots and 1½-section plots take up three times as much space. This raises the question of the relative efficiency of ½-section plots, full-section plots, and triple plots when the same area is used in each case. Immer [7] gives a method of determining the efficiency of plots of varying size and shape, calculated on the basis of variance per unit area of land, that can be used to answer this question. With the small plot as a standard, the relative efficiency of the larger plots was found by multiplying the square of the coefficient of variability per plot by the quotient of the area of the larger plot divided by the area of the small plot and expressing the result in percentage of the

[7] IMMER, F. R. SIZE AND SHAPE OF PLOT IN RELATION TO FIELD EXPERIMENTS WITH SUGAR BEETS. Jour. Agr. Research 44: 649-668, illus. 1932.

square of the coefficient of variability of the small plot used as a standard. When calculated in this way, if the ½-section plot is considered as 100 percent efficient it becomes apparent that on an area basis the efficiency of the full section is only 80.3 percent and the efficiency of the 1½-section plot only 68.3 percent. In other words, under the conditions exemplified by the plots studied, 10 replications of ½-section plots are preferable to 5 replications of entire sections; and 15 replications of ½-section plots are preferable to 5 replications of 1½-section plots.

Theoretically it seems probable that the customary method of filling the beds with compost and of using sections in the beds as units for filling would cause a greater variability between normal sections than between areas of similar size that do not coincide with the sections on the bed. The data at hand offer a means of testing this hypothesis. It is merely necessary to shift over one plot before pairing the yields of the ½-section plots to obtain for consideration a set of yields from areas equal in size to sections but overlapping the normal sections in the bed. If the foregoing conjecture is correct the coefficient of variability calculated from these units of area should be lower than the coefficient of variability calculated when normal sections are used as plots. An analysis of variance based on 4 shifted plots in each of 5 beds, as shown graphically in figure 2, gave a coefficient of variability of 9.57 in contrast to the coefficient of variability of 13.6 obtained for normal sections. The conclusion, therefore, seems justified that less variability may be expected when the experiment is so arranged that the areas used as units for harvesting do not coincide with the areas used as units for filling the beds with compost. On an area basis the overlapping plots were 201.9 percent as efficient as the normal sections and 162.2 percent as efficient as the ½-section plots.

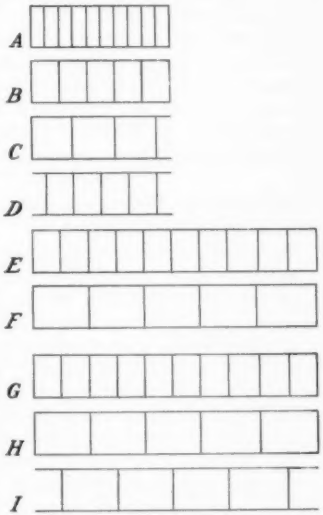

FIGURE 2.—Diagram showing relative size and arrangement of plots in typical blocks. *A–D*, Plots at Arlington Experiment Farm, Rosslyn, Va.: *A*, 2-foot plots, 10 plots per block (or bed), 5 blocks (total area); *B*, 4-foot plots, 5 plots per block, 5 blocks; *C*, 6-foot plots, 3 plots per block, 5 blocks; *D*, 4-foot plots, 4 plots per block, overlapping units of filling, 5 blocks. *E* and *F*, plots at Downingtown, Pa.: *E*, 4-foot plots, 10 plots per block, 5 blocks; *F*, 8-foot plots, 5 plots per block, 5 blocks. *G–I*, plots at Coatesville, Pa.: *G*, 4-foot plots, 10 plots per block, 4 blocks; *H*, 8-foot plots, 5 plots per block, 4 blocks; *I*, 8-foot plots, 4 plots per block, overlapping units of filling, 4 blocks.

DOWNINGTOWN, PA.

The yields of 50 plots from the five experimental beds at Downingtown, Pa., are given in table 1.

When the analysis of variance summarized in table 2 is applied to these data the value of z obtained from a comparison of the variance within beds with the variance between beds is 0.47468. This is slightly less than the 5-percent point, and indicates that, unlike the Arlington Farm experiments, the precision of the experiment was not

significantly increased by the arrangement of the plots to facilitate elimination of the variability between beds. In like manner it was shown that there is no justification for arranging the experiment in Latin squares and removing variability due to columns. On an area basis, however, there can be little doubt of the advantage of using replicate small plots rather than entire beds.

The coefficient of variability, calculated from the total variance for the data obtained by harvesting plots coinciding with sections on the bed (table 2), is 19.7. When the area of the plot is doubled (fig. 2 and table 2) the coefficient of variability drops to 14.8. The lower coefficient of variability is to be expected both on the basis of the increase in the size of plot and the dissimilarity of the area used for harvesting and the area used for filling the bed with compost. However, on an area basis the double section is only 88 percent as efficient as the section plot.

COATESVILLE, PA.

The yields of 40 plots from the 4 experimental beds at Coatesville, Pa., are given in table 1.

An analysis of variance applied to these data leads to conclusions similar to those derived from the experiments at Downingtown. Here again it is evident that small plots are preferable to entire beds on an area basis although there was no significant gain in the precision of the experiment by accounting in the analysis for the variance between beds or between columns. The coefficients of variability calculated from the total variance for single sections and double sections, respectively, were 10.6 and 7.2 (table 2). This experiment differed from the previous ones in that on an area basis the double section was slightly more efficient (108 percent) than the single section.

It should be recalled that in this experiment the units used for filling the beds with compost were double sections corresponding to plots 1 and 2, 3 and 4, 5 and 6, etc., in table 1. Theoretically the coefficient of variability should be lower if the yield data are combined so that the double sections used for plots in harvesting do not coincide with the areas used as units for filling the beds. To test this conjecture the coefficient of variability was calculated from the double sections 2 and 3, 4 and 5, 6 and 7, etc., as plots. With plots arranged in this way the coefficient of variability drops to 4.1. This corroborates the evidence in the Arlington experiments in favor of arranging the plots so as not to coincide with areas used as units in filling the beds. On an area basis double plots harvested in this manner are 308 percent as efficient as the double plots harvested from the same areas as were used for filling the beds and 334 percent as efficient as the single sections.

DISCUSSION AND CONCLUSIONS

The foregoing experiments demonstrate conclusively that small plots are preferable to entire beds as experimental units in a mushroom house. The variance of the small plots was in all cases less than the variance of entire beds. Furthermore, the small plots permit a greater number of yield comparisons on the same area and also permit increased precision through replication and through the arrangement of the plots so as to reduce the effect of compost heterogeneity and account for the variability between beds.

The results of the uniformity trials at both Arlington and Coatesville indicate an important relationship between the arrangement of the plots and the method employed for filling the beds with compost. When areas were used for harvesting which were comparable in size to the areas used for filling the beds but which overlapped the latter areas there was markedly less variability than when the same areas were used as units for both filling and harvesting. It would seem from this that the customary method of filling the beds by emptying 10 or 12 bushel baskets of compost at a time in each section or by emptying 20 or 24 bushels into each double section induces an excessive variability in the yields from these areas. It is advisable, therefore, to arrange the plots so that they do not coincide with the units of area used for filling the beds. Probably a further gain can be made in the precision of yield tests by modifying the system of filling experimental beds in order to mix the compost and distribute it more uniformly in the beds. A practical method of doing this is to fill the beds by emptying 1 bushel of compost at a time in each section at random in the bed until every section has received the required amount of compost.

This method of mixing the compost in the bed should reduce the variability between plots on a bed, but probably would have little or no effect on the variability between beds. Therefore it is necessary to account for the variability between beds in the reduction of the data. This may be accomplished by randomizing all the treatments on each of the beds so that the beds can be considered as replicate blocks in Fisher's randomized-block system of analyzing the data. As there was no significant fertility gradient (column effect) from one end of the house to the other, randomized blocks are preferable to Latin squares in that they leave more degrees of freedom for error and thus permit a more precise test of significance.

The question of the most desirable size among small plots is somewhat problematic. In all the trials the larger plots varied less than the smaller plots, so that more precision can be expected from the use of whole sections than ½-section plots, double sections than single sections, etc. But in 2 out of the 3 experiments the increase in precision with increase in plot size was not proportional to the increase in plot size. In other words, greater experimental precision was obtained for a given area by increasing the replication of small plots than by increasing the size of the plots. In practice, however, it is usually more expensive to increase replication than to increase plot size; so the experimenter must reach a compromise, depending on circumstances. In the writer's opinion sections 4 by 5 feet make satisfactory plots in a small experimental house such as the one at Arlington Farm and double sections are a good compromise for conventional houses. This allows the comparison of five different varieties or treatments in a single experiment if all the treatments are laid out on each of the replicate beds in accordance with the randomized-block system.

Before leaving the question of plot size, perhaps a word of explanation should be offered for an apparent anomaly in the results, namely, that increased precision is obtained by increasing the size of the small plots on the bed yet when entire beds are used as plots the experiments are less precise. Practical experience suggests that this is due to the fact that greater differences may be expected in the

moisture content of the compost, diseases, insect flora, and temperature from the top of the house to the bottom than from end to end. This is substantiated by the greater variation between beds than between columns shown in table 2.

The advisability of replicating treated plots need hardly be discussed in view of the fact, already pointed out, that on an area basis in 2 out of the 3 uniformity trials precision was gained more rapidly through replication than through increase in plot size. The question at issue is how many replications are advisable under the limitations laid down by the conditions of the experiment, the funds available, and the structural features of the experimental house. In the writer's opinion 5 or 6 replications are a good compromise since they will usually enable the investigator to detect differences in yield of approximately 10 percent, due to experimental treatment. If greater precision is necessary it can be obtained through increased replication, as the randomized-block plan allows as many replications as there are beds in the house—usually 12 to 20. When different composts are being compared it is desirable to replicate the compost heaps in addition to replicating the plots derived from each experimental compost heap.

SUMMARY

Yield data are discussed from uniformity trials in the experimental mushroom house at Arlington Experiment Farm, Rosslyn, Va., and in commercial houses at Coatesville and Downingtown, Pa.

These tests indicate that small plots are preferable to beds as experimental units. Double sections containing from 40 to 48 square feet of bed space seemed to be the most practical size of plot for experiments in commercial houses. In one of the experiments there was a distinct advantage in arranging the plots on the beds so as to make it possible to account for the excess variation between beds in analyzing the data.

A gain in precision also was apparent when test plots were so arranged that they did not coincide with the areas used as units for filling the beds. Therefore, a modification of the usual method of filling beds is suggested for experimental yield tests.

At least 5 or 6 replications seem to be necessary.

MAGNESIUM, CALCIUM, AND IRON REQUIREMENTS FOR GROWTH OF AZOTOBACTER IN FREE AND FIXED NITROGEN [1]

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INTRODUCTION

Most of the investigations concerning the inorganic nutrient requirements of *Azotobacter* have not involved comparisons of growth in the presence and absence of fixed nitrogen. The majority have been chiefly qualitative studies in which free nitrogen gas only was used, and they have yielded no evidence as to whether a particular element played a necessary role in fixation or was a general growth nutrient. Burk and Lineweaver (*6*) [2] have recently reported an elaborate series of experiments with both macrocultures and microcultures (Erlenmeyer and Warburg technic) in which calcium (or strontium) was the only metallic ion found to be specifically required in the catalysis of the nitrogen-fixing process at concentrations greater than 0.01 millimolal. Magnesium and iron (*7*) were found to be highly stimulating to the growth process in both free and fixed nitrogen over the respective concentration ranges 0.1 to 3 millimolal and 0.001 to 0.015 millimolal, but no studies were carried out at lower concentrations to ascertain whether these elements were strictly essential.

The present work has extended these investigations in a quantitative manner, with particular reference to the absolute growth requirements. Considerably modified cultural conditions have been employed. The experiments have lasted several days or weeks, instead of 6 to 48 hours, thereby permitting maximum growth. The nutrient media have been maintained at the most recently determined optimum concentrations with respect to all known nutrient requirements except the element or elements under consideration.

REVIEW OF LITERATURE

According to Buchanan and Fulmer (*5*), magnesium is essential to the growth of many, though not all, micro-organisms. Linossier (*22*) has shown that it is required for the development of *Oidium lactis*, Buromsky (*11*) that it is essential for the growth of *Aspergillus niger* and not replaceable by calcium, and Lockemann (*23*) and Frouin and Guillaumie (*13*) that it is not replaceable by calcium, strontium, or any of the rare earth metals in the case of the tuberculosis bacillus. Frouin and Ledebt (*14*) have shown magnesium to be replaceable by certain of the rare earths for the production of pigment by *Bacillus phocaneus*. Many others report stimulating action upon micro-organisms at low concentrations of magnesium, with toxicity as the concentration is increased to 100 to 500 millimolal; however, essentialness at very low concentrations has rarely been definitely determined.

[1] Received for publication Jan. 25, 1934; issued July 1934.
[2] Reference is made by number (italic) to Literature Cited, p. 994.

Rippel and Stoess (*27*) concluded from experiments with a variety of bacteria and fungi, as well as from a review of the literature, that calcium is not a generally indispensable nutrient for micro-organisms but is occasionally necessary for some special physiological process, such as nitrogen fixation by *Azotobacter*. On the basis of his comprehensive experiments concerning the calcium requirements of living forms, Loew (*24*) concluded that most lower organisms, in contrast to the algae and higher organisms, rarely contain calcium.

Krzemieniewska (*21*), who has made one of the few quantitative studies of the inorganic nutrient requirements of *Azotobacter*, found that 0.16 millimolal of magnesium and 0.12 millimolal of calcium were necessary for maximum growth, whereas zero concentrations of either substantially prevented all growth and nitrogen fixation. The experiments were carried out in free nitrogen only, however, so that a comparison of the action of either element on the growth and fixation processes was not possible. Schröder (*29*) found calcium not to be essential for *Azotobacter* growth in fixed nitrogen, but stimulating, and concluded that this effect was due more to a neutralizing action than to any specific effect of the calcium ion. Stapp and Ruschmann (*30*) found little or no beneficial effect of calcium in a medium containing nitrate.

Very little work has been carried out on the iron requirements for bacterial growth because the limiting concentration range is usually about one-thousandth that of elements such as magnesium or calcium, and most culture media, especially those containing organic nutrients, generally contain sufficient impurity to produce maximum growth. A brief review of the most pertinent previous investigations on general bacterial growth is given by Buchanan and Fulmer (*5, p. 411*). Previous work with respect to *Azotobacter* has been reviewed elsewhere (*7, pp. 441–442*).

METHODS

Several media were employed, termed "basal" (A), "customary" (B), "altered" (C), "humate-free" (D), and "charcoal-treated" (E). The basal medium A consisted of 0.64 g (3.68 millimolal) K_2HPO_4; 0.16 g (1.18 millimolal) KH_2PO_4; 0.2 g (0.81 millimolal) $MgSO_47H_2O$; 0.05 g (0.29 millimolal) $CaSO_42H_2O$; 0.2 g (3.42 millimolal) NaCl; 0.5 mg (0.009 millimolal) Fe as synthetic humate iron [3]; and 20 g (55.5 millimolal) Mallinckrodt's sucrose crystals per liter. This medium was used as a standard control in most of the work. It provided a heavy growth of *Azotobacter* (20 mg organic nitrogen per 100 cc) and did not precipitate upon being sterilized. Customary medium B, employed previously (*6, 7*), contained 1 percent glucose instead of 2 percent sucrose, no humate iron, and slightly more calcium and phosphate. Altered medium C contained more or less added Ca or Mg than basal medium A; it precipitated upon standing if the Mg content were lowered to 0.04 millimolal or the Ca or phosphate content increased 20 percent or more. Humate-free medium D consisted of basal medium A with no Fe added as humate iron. Charcoal-treated medium E was prepared by shaking 1 l of humate-free medium D with 75 g of purified Baker's animal charcoal, allowing to stand for several days, and filtering. Humate iron was added to the filtrate before use in order to insure optimum iron for growth.

[3] For method of preparation see the following publication: Horner, C. K., Burk, D., and Hoover, S. R. The preparation of humate iron and other humate metals. Plant Physiol. (In press.) 1934.

Preliminary purification of the charcoal in order to remove considerable amounts of adsorbed gases and other impurities was accomplished by leaching 3 to 6 times at intervals of several hours with equal weights of 6 N HCl and finally washing on a Buchner filter with dilute alkali and then distilled water until the wash water was neutral.

Fixed nitrogen when employed was added at the rate of 100 to 200 mg (7.1 to 14.2 millimolal) per liter, as KNO_3, NH_4Cl, or urea.

Erlenmeyer or Florence flasks of 150 to 250 cc capacity containing 15 to 25 cc of nutrient solution and plugged with cotton were employed as culture flasks. Sterilization was accomplished by autoclaving for 10 minutes at 15 pounds pressure, 20 percent extra distilled water being added to each flask to make up for loss by evaporation. The culture flasks were inoculated with 3 drops of culture grown in aeration bottles containing 100 cc of customary medium. In the experiments where it was desired to minimize as much as possible any Ca or Mg carried over by the inoculum, heavy 2- to 7-day cultures were diluted 50 to 100 times with sterile medium free from these elements. The cultures were incubated in duplicate or triplicate at 28° to 30° C. for 2 to 10 days, occasionally longer, with frequent observations.

Growth was measured turbidimetrically with a Bausch & Lomb nephelometer, corrections being made for any significant original turbidity in uninoculated cultures (cultures of low Mg content). The original and final pH values were measured colorimetrically by using the various La Motte indicators and standards. The original pH of all media was 7.0 ± 0.2. In general, the pH values tended to decrease a maximum of 1 to 2 units with increase in growth, except in the case of cultures with nitrate, where the values often increased. The buffering capacity of the medium was substantially independent of the Ca or Mg concentration, being controlled chiefly by the phosphate concentration. In general, the sugar was rarely entirely consumed by the end of an experiment.

The organism employed was *Azotobacter vinelandii*, capable of very active nitrogen fixation and long in use at this laboratory. According to the recent work of Kluyver and van Reenen (*18*), this species is the common motile form of *Azotobacter*, of relatively small size, occurring in soils. It produces a green fluorescent pigment, and at times assumes either a lighter, yellow, or darker, pink or purple, tinge, especially in the presence of molybdenum.

EXPERIMENTAL RESULTS

MAGNESIUM AND CALCIUM

Tables 1, 2, and 3 show fair examples of the relative growths obtained with *Azotobacter* with a wide variety of Mg and Ca concentrations under otherwise approximately optimal conditions. The ratios of growths in altered media C to those in basal medium A are given as the most direct means of comparing the results in the different experiments at various stages of growth and with the three forms of nitrogen employed. Table 1 shows the effect upon growth of lowering either Mg or Ca while the other element is kept constant at the usual concentration; the results with varying Mg in the 10-day experiment are plotted in figure 1.

In general, the fraction of normal growth at any concentration of Mg is roughly the same in free or fixed nitrogen, although the actual

growth may be 2 to 4 times as great in the latter. The fraction tends to become smaller with the duration of the experiment and extent of growth, as would be expected if Mg were an essential cell nutrient. In other experiments lasting 30 to 40 days, cultures without Mg were only about one-hundredth as turbid as the basal medium controls.

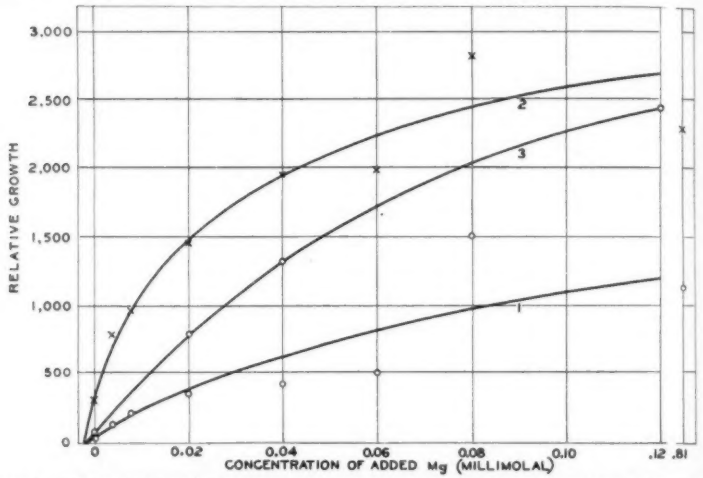

FIGURE 1.—Growth of *Azotobacter* in free and fixed nitrogen as a function of magnesium concentration. Curves 1 and 2, growth (turbidity) in N_2 and nitrate, respectively, 10-day experiment (table 1). Curve 3, growth (nitrogen fixed) in N_2, 12-day experiment, data of Krzemieniewska (*21*) (ordinate scale different from that for curves 1 and 2).

The growth of *Azotobacter*, independently of the source of nitrogen, decreases from normal at 0.05 to 0.1 millimolal Mg to one-fiftieth to one-hundredth of normal at 0.002 millimolal Mg (estimated maximum impurity in basal medium and inoculum).

TABLE 1.—*Effect of Ca and Mg concentrations upon Azotobacter growth and fixation*

Ca	Mg	Growth (turbidity) ratio, [a] altered medium C / basal medium A in—					
		Experiment 1, 3 days			Experiment 2, 10 days		
		N_2 (air)	KNO_3	Urea	N_2 (air)	KNO_3	Urea
Millimolal	*Millimolal*						
[b] 0.29	[b] 0.810	1.000	1.000	1.000	1.000	1.000	1.000
.29	.081	1.000	.573	.940	1.320	1.240	1.300
.29	.061	.356	.382	.658	.450	.873	1.270
.29	.040	.339	.339	.439	.367	.855	.795
.29	.020	.339	.329	.387	.306	.640	.306
.29	.008	.319	.329	.327	.181	.423	.183
.29	.004	.319	.267	.204	.122	.350	.144
.29	.000	.257	.114	.084	.037	.141	.066
.15	.810	.808	.958	.800	.718	.953	1.120
.06	.810	.628	1.040	.648	.768	1.050	1.220
.03	.810	.532	.840	.550	.568	1.295	1.190
.006	.810	.172	.710	.523	.437	.940	1.060
.00	.810	.130	.545	.470	.295	.606	1.190

[a] Relative growths in basal medium A—N_2: NO_3:urea (3 days) = 1:3.7:3.7; (10 days) = 1:2.0:4.1.
[b] Basal medium A.

TABLE 2.—*Effect of variable Ca-Mg ratios upon Azotobacter growth and fixation*

Ca	Mg	Ca/Mg	Growth (turbidity) ratio,[a] altered medium C / basal medium A in—								
			Experiment 1, 4 days			Experiment 2, 7 days			Experiment 3, 10 days		
			N_2(air)	KNO_3	Urea	N_2(air)	KNO_3	Urea	N_2(air)	KNO_3	Urea
Millimolal	*Millimolal*										
0.290	0.0	∞	0.162	0.628	0.230	0.101	0.315	0.093	0.032	0.332	0.073
.029	.0	∞	.085	.424	.280				.069	.202	.085
.012	.0	∞				.016	.175	.074			
.290	.081	3.58	1.220	.695	1.565				.981	.935	1.010
.012	.016	.75				.015	.589	.448			
.006	.008	.75				.018	.438	.214			
[b] .290	[b] .810	.36	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
.087	.240	.36	.918	.742	1.190				.705	.565	1.000
.029	.081	.36	.166	.935	.878				.158	.935	.885
[c] .004	[c] .028	.14	.130	.653	.638	.009	.667	1.000	.016	.556	1.030
.029	.81	.036	.695	.910	.878				.276	.764	.788
.029	2.43	.012	.855	1.125	.553				.329	.658	.963
.029	4.86	.006	.618	1.125	.807				.287	.633	1.030
.0	4.86	.0	.297	.963	.745				.158	.568	.980
.0	2.43	.0	.364	1.030	1.090				.333	.475	.878
.0	.81	.0	.173	.910	1.150	.164	.642	.972	.142	.424	1.050
.0	.081	.0	.067	.963	.807				.037	.742	.953
.0	.016	.0				.020	.547	.396			
.0	.0		.068	.454	.214	.013	.317	.108	.010	.263	.089

[a] Relative growths in basal medium A—N_2:NO_3:urea (4 days) = 1.0:2.7:4.8; (7 days) = 1.0:1.4:2.1; (10 days) = 1.0:0.9:1.5.
[b] Basal medium A.
[c] Charcoal-treated medium E.

TABLE 3.—*Influence of Ca in medium and inoculum upon growth in free and fixed nitrogen*

Nitrogen source	Ca in medium	Ca in inoculum	Growth (turbidity) in		
			2 days	3 days	6 days
N_2	[a] +	[a] +	.....	37	123
	+	−	.....	36	107
	[b] −	[b] +	.....	7	24
	−	−	.....	6	26
KNO_3	+	+	58	121	234
	+	−	56	92	227
	−	+	25	65	286
	−	−	25	75	149
Urea	+	+	44	212	484
	+	−	34	172	374
	−	+	43	200	472
	−	−	29	121	455
NH_4Cl	+	+	88	166	231
	+	−	52	141	231
	−	+	81	83	123
	−	−	60	100	140
Average of KNO_3, urea, and NH_4Cl	+	+	63	167	316
	+	−	47	135	277
	−	+	50	179	293
	−	−	38	99	248

[a] Basal medium A.
[b] Altered medium C (basal medium A without Ca). KNO_3 present in Ca-free medium used to prepare inoculum; such inoculum grown for 3 transfers previous to use.

When the Ca of the medium is lowered, a different relationship is seen to exist. Whereas the growth in fixed nitrogen may be decreased a maximum of 50 percent, that in free nitrogen is lowered 70 to 90 percent when but traces of Ca are present. Ca deficiency is more pronounced in the early stages of growth in both free and fixed

nitrogen. As growth progresses the need for Ca in fixed nitrogen is practically nullified; and, whereas a definite effect remains in free nitrogen, after a sufficient time (30 to 40 days) mere traces of Ca will permit 25 to 50 percent of normal growth. This substantiates the conception that Ca acts in a truly catalytic role in nitrogen fixation; only traces are absolutely necessary provided sufficient time is allowed, but relatively high concentrations are required to produce a maximum rate of fixation. The Ca needed for normal maximum growth in free nitrogen is 0.15 to 0.30 millimolal or practically saturation (0.36 millimolal), and this range appears to hold also in the early stages of growth in fixed nitrogen, but decreases after several days to less than 0.01 millimolal in nitrate and presumably to zero in urea (and ammonia).

The decrease with the duration of the experiment in the Ca requirements for growth in fixed nitrogen is indicated in table 3. The culturing of the inoculum for several previous transfers in Ca-free medium does not enable the organisms to become adapted to such a medium since somewhat less growth is obtained, as compared to inoculum grown in a medium containing calcium. This is best seen in the "average" values and tends to indicate a very small but definite Ca requirement in fixed nitrogen for entirely maximum, as distinguished from approximately maximum, growth. It is seen that the Ca requirement in free nitrogen remains considerable even after 6 days, the ratio of growth in its presence and absence (except for impurities) being 6 to 1 at 3 days and 4½ to 1 at 6 days.

In table 2 are given the growth ratios with a variety of Ca/Mg ratios principally under suboptimal concentrations of both elements. The most definite results are obtained with the older cultures. It is seen that little if any effect can be attributed to the Ca/Mg ratio, as has been reported frequently with supraoptimal concentrations with various organisms in connection with antagonism (*5, pp. 282–284, 356–359*). The independent effect of each element can be analyzed. Varying the Ca in the absence of Mg has very little effect in any case, since the lack of Mg is already limiting the growth almost completely. The two 0.75 Ca/Mg ratios show no significant difference in N_2, the Ca and Mg both being highly limiting; in fixed nitrogen the decrease corresponds to the Mg decrease. The three 0.36 ratios, with widely varying concentrations of the two elements, show definite decreases of growth in N_2 (due to calcium deficiency), but relatively little in fixed nitrogen. Keeping the Ca constant at 0 or 0.029 millimolal and increasing the Mg sixfold from 0.81 to 4.86 millimolal causes no consistent significant change in the amount of growth. As indicated previously, toxic effects are obtained at concentrations much greater than 4.86 millimolal (6 "customary").

The charcoal treatment of basal medium A was developed to remove, if possible, some nutrient or nutrients specifically required in growth or fixation, either in large or very small concentration. Hopkins (*17*) used this method (without the charcoal purification) to remove traces of iron from dissolved sugar. As shown in table 2 (Ca/Mg = 0.14), the effect of the treatment can be attributed almost entirely to the Ca and Mg removed; the Ca was found upon analysis, by an oxalate precipitation method, to have been reduced to about one-seventieth, and the Mg, by an ammonium phosphate precipita-

tion method, to one-thirtieth of the original concentrations. The Mg retained is still sufficient to permit 50 to 100 percent normal growth in fixed nitrogen, whereas in free nitrogen the combined Mg-Ca deficiency permits but slight growth, owing chiefly to lack of Ca and secondarily to lack of Mg; the effect of Mg deficiency is enhanced at low Ca. The average growth ratios in the charcoal-treated medium E for seven different 6- to 10-day experiments were: N_2, 0.015; KNO_3, 0.595; urea, 0.715. As may be seen in table 2, these growth ratios are approximately those obtained when the same order of Ca and Mg concentrations are added intentionally in making up altered media.

The preponderant influence of Ca-Mg deficiency in the charcoal-treated medium E was also demonstrated by determining the effect of adding thereto the different basal medium A salts separately. The KH_2PO_4, K_2HPO_4, and NaCl caused substantially no improvement. Humate iron was normally added to all charcoal-treated medium E, but special experiments involving its omission showed that it likewise was not beneficial. Table 4 shows the effect of adding Mg and Ca separately and together to the charcoal-treated medium E. The most striking results, as would be expected, are observed in free nitrogen. Ca alone increases the growth about 7 times, Mg about 18 times, and both together about 45 times, or to within 25 percent of that in basal medium A. The fact that Ca and Mg together did not cause complete recovery indicates that some other slight inhibiting factor may be involved in the charcoal treatment, but a 10- to 30-percent variation can be expected in the methods of culturing and measurement employed. Since the deficiency in the case of fixed nitrogen is not sufficient to cause more than this amount of inhibition, no definite beneficial effect of the added Ca and Mg can be detected. Neither the Ca nor the Mg alone is sufficient to return the growth to normal since the other element is always deficient. When either Mg or Ca solutions (of basal medium A concentration) are treated with charcoal and added to the other salts untreated, both media so obtained show growth inhibition, as would be expected. This inhibition is overcome by adding Mg or Ca, respectively. Similar treatment of phosphate or NaCl solutions yields no inhibition. It may be stated also that the extent of reduced growth obtained in charcoal-treated medium E was independent of the concentration of Fe added and also the amount or age of inoculum. Finally, sugar charcoal as compared with animal charcoal yielded no growth reduction; in fact it was observed to provide iron in media deficient in this element.

TABLE 4.—*Growth recovery in charcoal-treated medium E upon addition of Mg or Ca*

Medium	Growth (turbidity) ratio,[a] $\frac{\text{altered medium C}}{\text{basal medium A}}$ when source of nitrogen was—		
	N_2 (air)	KNO_3	Urea
Basal A	1.000	1.000	1.000
Charcoal-treated E	.016	.787	.708
Charcoal-treated E+0.29 millimolal Ca	.115	.805	[b].565
Charcoal-treated E+0.81 millimolal Mg	.297	[b].595	.845
Charcoal-treated E+0.29 millimolal Ca and 0.81 millimolal Mg	.732	.787	.808

[a] Relative growths in basal medium A—N_2:NO_3:urea (6 days)=1.0:1.0:1.7.
[b] Low.

The apparent essentialness of Mg for *Azotobacter* growth, as observed in the preceding experiments, is almost certainly a true magnesium requirement. The Baker's $MgSO_4.7H_2O$ employed was examined spectroscopically.[4] Only sodium was found in considerable amount, with definite but faint indication of aluminum, copper, calcium, iron, lithium, and silicon, and possibly vanadium and boron (no potassium). All these elements, and others, were tested for their effect in either charcoal-treated medium E or basal medium A containing no Mg. The following metal ions were added to charcoal-treated medium E: Cu, Mn, Ni, Co, Al, Zn at 0.1 p.p.m.; Mo, Si, Ti, Cr at 1 p.p.m.; V, Mn, B, Li, and Ba at 0.1 and 1 p.p.m.; 20 and 200 p.p.m. of natural humic acid, a material which undoubtedly contains traces of all basic elements normally found in soil; and also 10 p.p.m. coenzyme R (*1*). No increase in growth was observed with any of these. In the magnesium-free medium the following metals were tested: V, B, Mo, and Li at 2.5 and 10 p.p.m.; Ba and Sr at 2.5, 10, 25, and 50 p.p.m.; and Mn at 0.5, 2.5, 10, and 12.5 p.p.m. Mn, Ba, and Sr appeared to be slightly beneficial. The other elements yielded no increase in growth. Mg as such is therefore essential with no element able to act very efficiently in place of it. No stimulation in basal medium A resulted from 0.1 and 1 p.p.m. Cu, Al, Ni, and Mn, but stimulation was often increased 1½ to 3 times by either V (VCl_3, Na_3VO_4, or $K_2V_4O_9$) or Mo ($NaMoO_4 2H_2O$) at 1 p.p.m., in agreement with previous findings of Bortels (*3*, *4*), Burk and Lineweaver (*6*), Birch-Hirschfeld (*2*), Schröder (*29*), Kluyver and Van Reenen (*18*), and Konishi and Tsuge (*19*).

IRON

The problem of demonstrating a definite iron requirement usually necessitates purification of the medium rather than the addition of iron, owing to the low values involved. The phosphate-adsorption method reported by Hopkins (*17*) in connection with the green alga *Chlorella*, and the carbonate treatment described by Steinberg (*31*) in connection with the bread mold *Aspergillus*, for removing traces of Fe were found to be incapable of causing any Fe deficiency for *Azotobacter* in basal medium A without humate iron (medium D). There still remained after treatment 0.001 to 0.003 millimolal Fe. The modified Hopkins' charcoal treatment employed for removal of Mg and Ca masked any possible removal of Fe. Ruhland's (*28*) method of autoclaving and filtering a nutrient solution composed only of inorganic nutrients was found to lower the Fe impurity of the inorganic salts of basal medium A but could not be employed for sugar purification. Several sugars were, therefore, obtained, which when analyzed by a method previously described (*7*, *table 1*), showed a varying and relatively low Fe content, as follows: Difco dextrose, 4×10^{-4} percent; Merck's dextrose, 6×10^{-5} percent; Baker's sucrose, 6×10^{-5} percent; and Mallinckrodt's sucrose, 9×10^{-6} percent. The inorganic basal medium A (without humate iron or sugar) contained before and after autoclaving and filtering while hot 2.6×10^{-5} millimolal and 1.7×10^{-5} millimolal Fe, respectively. It was possible to obtain 8 Fe concentrations ranging from 5×10^{-5} millimolal to 4.5×10^{-4} millimolal by employing each of the 4 sugars at 13.9 millimolal (0.5 percent) together with 41.6 millimolal (1.5 percent) of the

[4] These examinations were made by the Bausch & Lomb Optical Co.

Mallinckrodt's (very low iron) sucrose, basal inorganic medium A being used with and without 5×10^{-5} millimolal humate iron (Fe impurity in synthetic humic acid with no Fe intentionally added); the lowest concentration was obtained by using 55.5 millimolal (2 percent) Mallinckrodt's sucrose in inorganic medium previously autoclaved and filtered. Maximum iron was assured by adding 1.8×10^{-3} millimolal humate iron to some of the cultures.

Figure 2 shows the relative growths obtained with these varying Fe concentrations. The curves definitely approach zero growth at the lowest concentrations, whereas the sugar, Difco dextrose, with the maximum Fe impurity, 4×10^{-5} millimolal, yields practically normal growth. The concentration of Fe yielding maximum growth increases somewhat with age of culture (curves 1 and 1*a*, 2 and 2*a*), as was shown previously in a different manner in connection with humic

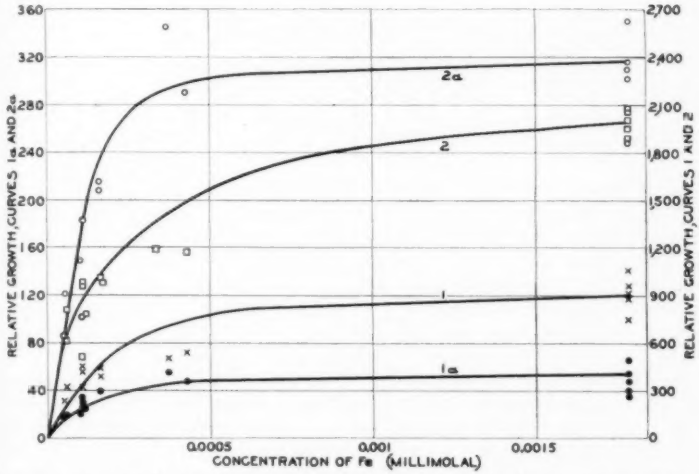

FIGURE 2.—Growth of *Azotobacter* in free and fixed nitrogen as a function of iron concentration: Curves 1 and 2, growth (turbidity) in N_2 and nitrate, respectively, 9-day experiment; curves, 1*a* and 2*a*, 2-day experiment.

acid stimulation (*8, p. 458, fig. 2*). At any given age of culture approximately the same differences in amount of growth in free and fixed nitrogen are obtained at all iron concentrations (table 5), indicating that, as determined by this method, there was no specific fixation effect of Fe deficiency, such as was obtained so definitely in the case of Ca. The phenomenon of the growth in free nitrogen approaching that in fixed nitrogen, after a prolonged period of time, is shown by the "average" values, table 5, and by comparing the ordinate ranges between curves 1 and 2 and curves 1*a* and 2*a*. This phenomenon is commonly observed and presumably bears no direct relation to Fe requirements, but to limitations by other factors (*8, pp. 455–462*). Ruhland (*28*) has reported a limiting Fe concentration range for the autotrophic hydrogen organism *Bacillus pycnoticus* which is essentially the same as that demonstrated for *Azotobacter*, no growth being obtained at 10^{-5} millimolal and maximum growth occurring by 1.2×10^{-3} millimolal.

TABLE 5.—*Ratio of growth in fixed and free nitrogen at different iron concentrations*

Concentration of Fe (millimolal)	Growth (turbidity) ratio, $\frac{KNO_3}{N_2}$ for experiment—			
	No. 1[a]		No. 2	
	2 days	9 days	3 days	5 days
4.8×10^{-5}	5.0	2.7		
5.9×10^{-5}	6.7	2.6		
1.01×10^{-4}	5.5 (3.4, 6.0, 7.0)	2.3 (2.3, 2.2, 2.4)		
1.1×10^{-4}	7.0	2.4		
1.6×10^{-4}	5.6 (5.4, 5.7)	2.4 (2.3, 2.5)		
4.1×10^{-4}	6.2	2.3		
4.6×10^{-4}	6.1	2.2		
1.8×10^{-3}	6.6 (5.4, 8.0, 3.8, 7.3, 8.7)	2.2 (2.0, 2.1, 2.0, 2.3, 2.6)		
1.8×10^{-2}	5.7 (5.8, 7.4, 2.7, 5.6, 7.1)	2.1 (1.6, 2.0, 2.6, 1.9, 2.4)		
2.5×10^{-5}			4.6	4.3
3.6×10^{-5}			3.0	3.5
8.1×10^{-5}			5.7	2.6
3.8×10^{-4}			4.3	3.3
1.8×10^{-3}			4.5 (4.3, 4.3, 5.3, 4.2)	3.1 (3.1, 2.9, 2.0, 4.4)
Average	6.1	2.3	4.4	3.4

[a] Compare fig. 2, p. 989.

COMPARISON OF ABSOLUTE ELEMENT REQUIREMENTS

Comparing the concentrations of essential elements producing half maximum growth ($C_{G/2}$) is as convenient, and more accurate, than comparing those required for optimum growth, under variable experimental conditions. Table 6 gives $C_{G/2}$ values for Ca, Mg, and Fe under various conditions of age of culture and nitrogen source, and also those for Ca and Mg determined from Krzemieniewska's data (*21*). These values have been determined graphically from curves such as those shown in figure 1 for Mg and in figure 2 for Fe. The values for Mg and Fe do not vary significantly with respect to nitrogen source, being essential elements in any case, whereas the nonessentialness of Ca in fixed nitrogen is strikingly shown by the lack of any definite positive value, as compared with 2×10^{-2} to 5×10^{-2} millimolal in free nitrogen. $C_{G/2}$ for Fe is about one five-hundredth of that for Mg or Ca in free nitrogen.

In general, $C_{G/2}$ should decrease with age or heaviness of cultures if the element is utilized relatively more efficiently at lower concentrations by being employed over and over again in a catalytic manner. It should increase if the element is consumed and prevented from further functioning. As can be seen from table 6, there are no changes in $C_{G/2}$, for any of the elements, great enough to distinguish conclusively between catalysis or consumption or a combination of both. A tendency appears for a decrease with Mg and Ca and an increase with Fe, and earlier evidence with Ca and Fe supports this interpre-

tation. $C_{G/2}$ probably varies relatively little for growths ranging from 2 to 20 mg organic nitrogen per 100 cc (light to very heavy growths, about 20 to 200 mg dry matter per 100 cc); it should be a fairly characteristic constant. This is important in connection with the basal medium A employed, since normally Mo and V were not added, although in experiments involving very heavy growth they could cause definite increases in growth.

TABLE 6.—*Approximate concentrations of calcium, magnesium, and iron required for half maximum growth of Azotobacter using various sources of nitrogen*

Element	$C_{G/2}$, the concentration (millimolal) yielding half-maximum growth when source of nitrogen was—							
	N_2 (air)		KNO_3		Urea		N_2 (Krzemieniewska (*21*))	
	2-3 days	9-10 days	2-3 days	9-10 days	2-3 days	9-10 days	5 days	12-15 days
Ca	4×10^{-2}	2×10^{-2}	*$0-2\times10^{-4}$	*$0-2\times10^{-4}$	*$0-2\times10^{-4}$	*$0-2\times10^{-4}$	8×10^{-3}	5×10^{-2}
Mg	6×10^{-2}	4×10^{-2}	6×10^{-4}	2×10^{-3}	5×10^{-2}	3×10^{-2}	5×10^{-2}	4×10^{-2}
Fe	1.1×10^{-4}	1.4×10^{-4}	1.1×10^{-4}	1.6×10^{-4}				

* Maximum Ca impurity in medium and inoculum.

Krzemieniewska (*21*) reported that potassium, sulphur, and phosphorus were required for *Azotobacter* growth in relatively large concentrations, the first-named at about that found for Ca and Mg, and the latter at a value 3 to 10 times as great. Vogel (*32*) and Stapp and Ruschmann (*30*) report, however, that the addition of potassium to media was not required. Similarly the present writers, and Stapp and Ruschmann (*30*), have found that the traces of sulphur occurring as impurities will yield at least 50 to 75 percent of maximum growth. The phosphorus requirements have been generally assumed to be relatively high; Burk and Lineweaver (*6*) have indicated that 0.1 millimolal is sufficient.

DISCUSSION

The experiments of long duration reported above are seen to confirm the previous observations (*6*) involving experiments essentially of short duration concerning the catalytic effect of Ca upon the nitrogen-fixation process of *Azotobacter*. The small concentrations of Ca studied in the present work yield still more evidence that Ca acts in a truly catalytic role. The relatively large concentrations emphasized previously (0.10 to 0.30 millimolal) applied to obtaining the maximum rate of fixation by young cultures, rather than to the maximum amount of growth after a long period of time. The apparent stimulation of growth in fixed nitrogen by Ca is much lessened, if not upon occasion entirely eliminated, as the duration of the experiment is increased.

The essentialness of Mg for the growth of *Azotobacter* is much more evident from the experiments of long duration, with the other inorganic nutrients at normal concentrations, than in the experiments of Burk and Lineweaver (*6*) where 10 times the customary diluted medium was chiefly employed. Whereas some growth may occur in free nitrogen with less than 2×10^{-3} millimolal Mg (estimated maxi-

mum Mg impurity) in the first 24 to 48 hours, surpassing that obtained with a medium containing 0.81 millimolal Mg, but no Ca, growth in the former practically ceases after a few days, whereas in the latter a definite increase occurs. In this case the Ca is being used continuously at very small concentrations.

As mentioned above, and observed in a former paper (*6, p. 164,* "pseudo-Ca-Sr-effect"), Mg appears to possess at times a somewhat, greater relative beneficial effect upon growth in free nitrogen than in fixed nitrogen, particularly at low concentrations of Ca. For the present, this is still interpreted as involving no specific influence upon the fixation process proper, but probably making any Ca present in the cells more available, perhaps merely more soluble. In this connection, Mg has been observed to interfere markedly with Ca precipitation by oxalate, and also, as mentioned above, serves to maintain a clear nutrient solution by increasing the solubility of calcium phosphate. This effect upon Ca solubility would scarcely be related to the chief effect of Mg in the growth process, since Ca is substantially unnecessary there.

Haines (*16*) has reported a somewhat parallel relationship with Ca and Mg between the production of bacterial gelatinase and growth with five different micro-organisms. Ca alone promotes the formation of the enzyme while permitting but poor growth; Mg alone yields good growth but substantially no gelatinase. The best effect is obtained in the presence of both elements, probably owing in part to the influence of growth itself upon the protease production. In a previous work, Haines (*15*) reported a similar effect with the enzyme attacking caseinogen. He suggests that future work might show salts of Ca to be essential for the formation of proteases in general. Although this view might appear contradictory to Rippel and Stoess' (*27*) conclusion as to the general unessentialness for Ca for micro-organisms, since the majority probably produce proteases, it suggests in connection with the present work with *Azotobacter* that Ca when essential for organisms is a constituent of some particular enzyme. Thus, Nakamura (*26*) has found that Ca is an essential activator for amylase. The concentration of Ca has recently been shown, however, to have no influence on two specific aspects of fixation by *Azotobacter*, namely, the pH limit at 6, and the Michaelis dissociation constant or nitrogen pressure at which half maximum velocity of fixation is obtained (*9*).

Mg may also act specifically as a constituent of certain frequently occurring enzymes, as well as in some general phenomenon such as permeability. Lohmann (*25*) has found that Mg is an essential constituent of the coenzyme for lactic acid formation in muscle and for cozymase in yeast, and Erdtman (*12*) has found that it is necessary for liver phosphatase. The general utilization of Mg in oxidative processes is improbable, however, because of their diversity in the various organisms. Kruse, Orent, and McCollum (*20*) have shown that Mg deficiency in young rats leads to death and that the most striking blood change is a disturbance of the lipoid distribution. This finding may disclose upon further investigation that Mg is concerned generally in some phase of lipoid behavior.

The observation that the iron requirement of *Azotobacter*, as measured by final amounts of growth obtained with the Erlenmeyer technic, is substantially independent of the source of nitrogen con-

firms the previous finding (*8, pp. 465, 482, 486; 10, p. 523*) that in experiments of this type the stimulation caused by iron supplied to customary medium, as humate or otherwise, is approximately the same in free and fixed nitrogen; that is, iron does not normally appear to influence directly or specifically the availability of either of these sources of nitrogen, the one more than the other. The very much lower concentrations of Fe as compared to Ca required in free nitrogen make a specific fixation requirement somewhat more difficult to determine by the methods here employed. In earlier papers reference was made (*7, pp. 427, 447; 8, p. 465 and table 6; 10, p. 523*) to unpublished work on the apparent specific effect of iron in fixation under certain particular conditions, obtained with the Warburg technic, where the velocity constants as distinguished from the final amounts of growth were observed. This apparent specific effect of iron has now been definitely determined to be due to an exceedingly small molybdenum impurity in the iron. The molybdenum is effective at a concentration of only 1 to 100 mg [4] per 1,000,000,000,000 mg medium (1 to 100 parts per trillion, or 10^{-11} to 10^{-9} molal Mo.)

The amounts of iron required for maximum growth of *Azotobacter* as determined in the experiments reported here are approximately the same as those indicated in previous work (*7*), namely, 0.0004 to 0.01 millimolal (0.02–0.5 p.p.m.). In the experiments described in table 5 and figure 2, the concentration 0.018 millimolal as compared to 0.0018 millimolal gave growths 10 and 22 percent higher, on an average, at 2 and 9 days, respectively. No similar comparison of $C_{G/2}$ values is possible, since it is only in the present work that the low concentrations of Fe have been studied.

SUMMARY

The magnesium, calcium, and iron requirements for growth of *Azotobacter vinelandii* in the presence of free and various forms of fixed nitrogen have been investigated quantitatively, and brief reference made to other inorganic elements. Various types of media were employed. In comparison with work reported previously, the experiments were of relatively long duration (2 to 10 days), and the growths obtained were very heavy (20 to 200 mg dry matter per 100 cc).

The concentrations of the respective elements yielding half maximum growth ($C_{G/2}$) have been determined as the most accurate means of making various comparisons. In these experiments $C_{G/2}$ was relatively independent of duration of experiment and extent of growth. $C_{G/2}$ for Ca is $2–5 \times 10^{-2}$ millimolal in free nitrogen, and negligible ($0–2 \times 10^{-4}$ millimolal) in fixed nitrogen, such as nitrate, ammonia, or urea; this difference confirms previous findings concerning the specific role of calcium in the nitrogen-fixing process. With Mg and Fe, $C_{G/2}$ is independent of the source of nitrogen, being $2–6 \times 10^{-2}$ and $1.1–1.6 \times 10^{-4}$ millimolal, respectively. The essential role of both Mg and Fe in growth is indicated by an approach to zero growth at the lowest concentrations. No specific requirement of Fe (or Mg) in fixation was evident in experiments of the type here employed, confirming previous findings that normally humate iron exerts the same stimulation in free and fixed nitrogen.

[4] BURK, D. AZOTASE AND NITROGENASE IN AZOTOBACTER. Review chapter in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforshung. Illus. Leipzig. 1934.

The concentrations of Mg, Ca (in free nitrogen), and Fe required for maximum growth are 0.05–0.1 millimolal, 0.1–0.3 millimolal, and 0.0004–0.001 millimolal, respectively. The requirement for P appears to be 0.1 millimolal, and for S, K, Mo, and V equal to or less than that for Fe.

Mg could not be replaced in the growth process by Cu, Mn, Ni, Co, Al, Zn, Ca, Sr, Ba, Mo, Si, Ti, Cr, V, B, or Li applied in various concentrations.

In ascertaining the very low Fe requirement, adsorption methods involving charcoal, calcium carbonate, or calcium phosphate did not suffice to free the medium from iron. It was necessary to select sugars with different and very low amounts of iron. This method should be useful in connection with various elements similarly needed in traces in general bacterial growth.

LITERATURE CITED

(1) Allison, F. E., Hoover, S. R., and Burk, D.
1933. A respiration coenzyme. Science (n.s.) 78:217–218.

(2) Birch-Hirschfeld, L.
1932. Über den einfluss von molybdän und bodenextraktstoffen auf die N-bindung von azotobacter chroococcum. Arch. Mikrobiol. 3:[341]–361, illus.

(3) Bortels, H.
1930. Molybdän als katalysator bei der biologischen stickstoffbindung. Arch. Mikrobiol. 1:[333]–342.

(4) ———
1933. Kurze notiz über die katalyse der biologischen stickstoffbindung. Centbl. Bakt. [etc.] (II) 87:[476]–477.

(5) Buchanan, R. E., and Fulmer, E. I.
1930. Physiology and biochemistry of bacteria. v. 2. Baltimore.

(6) Burk, D., and Lineweaver, H.
1931. The influence of calcium and strontium upon the catalysis of nitrogen fixation by azotobacter. Arch. Mikrobiol. 2:[155]–186, illus.

(7) ———, Lineweaver, H., and Horner, C. K.
1932. Iron in relation to the stimulation of growth by humic acid. Soil Sci. 33:413–452, illus.

(8) ——— Lineweaver, H. and Horner, C. K.
1932. The physiological nature of humic acid stimulation of azotobacter growth. Soil Sci. 33:455–487, illus.

(9) ——— Lineweaver, H., and Horner, C. K.
1934. The specific influence of acidity on the mechanism of nitrogen fixation by azotobacter. Jour. Bact. 27:325–340, illus.

(10) ——— Lineweaver, H., Horner, C. K., and Allison, F. E.
1931. The relation between iron, humic acid, and organic matter in the nutrition and stimulation of plant growth. Science (n.s.) 74: 522–524.

(11) Buromsky, J.
1912. Die salze, zn, mg, und ca, k und na und ihr einfluss auf die entwicklung von aspergillus niger. Centbl. Bakt. [etc.] (II) 36: 54–66.

(12) Erdtman, H.
1928. Über nierenphosphatase. iii. Hoppe-Seyler's Ztschr. Physiol. Chem. 177: [231]–236.

(13) Frouin, A., and Guillaumie, M.
1926. Nutrition minérale du bacille tuberculeux. Bull. Soc. Chem. Biol. 8: [1151]–1177.

(14) ——— and Ledebt, S.
1912. Action du vanadate de soude et des terres rares sur le développement du bacille pyrocyanique et la production de ses pigments. Compt. Rend. Soc. Biol. [Paris] 72: 981–983.

(15) HAINES, R. B.
1931. THE FORMATION OF BACTERIAL PROTEASES, ESPECIALLY IN SYNTHETIC MEDIA. Biochem. Jour. 25: [1851]–1859.

(16) ———
1933. FURTHER STUDIES OF THE EFFECT OF THE MEDIUM ON THE PRODUCTION OF BACTERIAL GELATINASE. Biochem. Jour. 27: [466]–474, illus.

(17) HOPKINS, E. F.
1930. IRON-ION CONCENTRATION IN RELATION TO GROWTH AND OTHER BIOLOGICAL PROCESSES. Bot. Gaz. 89: 209–240, illus.

(18) KLUYVER, A. J., and REENAN, W. J. VAN.
1933. UBER AZOTOBACTER AGILIS BEIJERINCK. Arch. Mikrobiol. 4: [280]–300.

(19) KONISHI, K., and TSUGE, T.
1933. EFFECT OF INORGANIC CONSTITUENTS OF SOIL SOLUTION ON THE GROWTH OF AZOTOBACTER. Jour. Agr. Chem. Soc. Japan 9: 129–144.

(20) KRUSE, H. D., ORENT, E. R., and MCCOLLUM, E. V.
1933. STUDIES ON MAGNESIUM DEFICIENCY IN ANIMALS. III. CHEMICAL CHANGES IN THE BLOOD FOLLOWING MAGNESIUM DEPRIVATION. Jour. Biol. Chem. 100: 603–643, illus.

(21) KRZEMIENIEWSKA, H.
1910. DER EINFLUSS DER MINERALBESTANDTEILE DER NAHRLÖSUNG AUF DIE ENTWICKLUNG DES AZOTOBAKTERS. Bull. Internatl. Acad. Sci. Cracovie Math. et Nat. 1910 (B): [376]–413.

(22) LINOSSIER, G.
1917. SUR LA BIOLOGIE DE L'OIDIUM LACTIS. INFLUENCE DE LA QUANTITÉ DES ALIMENTS MINÉRAUX SUR LE DÉVELOPPEMENT DU CHAMPIGNON. Compt. Rend. Soc. Biol. [Paris] 80: 433–435.

(23) LOCKEMANN, G.
1919. WELCHE NÄHRSTOFFE SIND FÜR DAS WACHSTUM DER TUBERKELBAZILLEN UMBEDINGT NOTWENDIG. Centbl. Bakt. [etc.] (I) 83: 420–425.

(24) LOEW, O.
1912. ÜBER DIE GIFTWIRKUNG VON OXALSAUREN SALZEN UND DIE PHYSIOLOGISCHE FUNKTION DES CALCIUMS. Biochem. Ztschr. 38: [226]–243.

(25) LOHMANN, K.
1931. ÜBER DAS KOFERMENT DER MILCHSÄUREBILDUNG DES MUSKELS. Naturwissnschaften 19: 180.

(26) NAKAMURA, H.
1931. AMYLASE PROTECTING SUBSTANCES. VII. THE IDENTIFICATION OF THE CHEMICALLY PURE PROTECTIVE SUBSTANCES. Jour. Soc. Chem. Indus. Japan Sup. Binding 34: 211–222.

(27) RIPPEL, A., and STOESS, U.
1932. IST CALCIUM EIN FÜR MIKROORGANISMEN NOTWENDIGES ELEMENT? Arch. Mikrobiol. 3: [492]–506.

(28) RUHLAND, W.
1924. BEITRÄGE ZUR PHYSIOLOGIE DER KNALLGASBAKTERIEN. Jahrb. Wiss. Bot. 63: [321]–389, illus.

(29) SCHRÖDER, M.
1932. DIE ASSIMILATION DES LUFTSTICKSTOFFS DURCH EINIGE BAKTERIEN. Centbl. Bakt. [etc.] (II) 85: [177]–212.

(30) STAPP, C., and RUSCHMANN, G.
1924. ZUR BIOLOGIE VON AZOTOBAKTER. Arb. Biol. Reichsanst. Land u. Forstw. 13: [305]–368.

(31) STEINBERG, R. A.
1919. A STUDY OF SOME FACTORS IN THE CHEMICAL STIMULATION OF THE GROWTH OF ASPERGILLUS NIGER. Amer. Jour. Bot. 6: 330–372.

(32) VOGEL, [J.].
1912. UNTERSUCHUNGEN ÜBER DAS KALIBEDÜRFNIS VON AZOTOBAKTER. Centbl. Bakt. [etc.] (II) 32: 411–421.

THE NORMAL DEVELOPMENT OF THE LEG BONES OF CHICKENS WITH RESPECT TO THEIR ASH CONTENT[1]

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INTRODUCTION

The ash content of certain bones, especially those of the legs, has been used in recent years as a measure of the effect of experimental diets on the mineralization of the skeleton of the animals to which the diets were fed. This has been particularly true in some of the nutrition studies with chickens, in which the percentage of ash in one or another of the leg bones has been used to determine the extent of the occurrence of rickets. The ease with which rickets may be produced in chickens has led to the use of chickens as laboratory animals for determining the antirachitic value of the various vitamin D supplements, particularly cod-liver oil, used in diets for poultry.

In order that a correct interpretation may be placed on the results of work of this nature, it is necessary that data on the ash content of the bones of birds reared under normal environmental conditions be available for the purpose of comparison. Furthermore, these data should furnish information on the effect, if any, of age, breed, and sex on the ash content of the bones. The published percentages of ash which have been considered as normal were obtained almost wholly from the analysis of the bones of birds reared under rather artificial conditions, and they represent only one or, at best, a few ages. It is necessary also to know how the several factors other than diet affect the deposition of the mineral elements in bone, for otherwise one cannot be certain that the results obtained are due only to the diets, or, more specifically, to the vitamin D supplements being studied.

Furthermore, it is often desirable to compare the results of work carried out in different laboratories, and in order that this may be possible it is necessary that comparable methods be used for the determination of the ash in the bones of the experimental birds. At present there does not seem to be any great degree of unanimity among the various investigators in this respect. In general the tibia has been used for this purpose, but in some cases other bones have been used. Some reports show different ways of preparing the bones for analysis; others show the use of different methods for ash determination. Some of these differences probably had little or no effect on the results obtained, but it is also probably true that some had a marked effect.

This lack of uniformity in the methods used for the determination of the ash of the leg bones, as well as the lack of adequate information regarding the percentage of ash in the bones of chickens reared under normal conditions, made it desirable to ascertain the ash content of the bones of such chickens of both sexes and of different ages and breeds by the use of a simple and rapid method.

[1] Received for publication Jan. 16, 1934; issued July 1934.

PLAN OF THE INVESTIGATION

It was planned to rear two groups of chickens, each group of a different breed, under favorable conditions on a grass range, and to study the normal development of their leg bones with respect to the ash content. Each week, for 20 weeks, representative birds were to be selected from each group to furnish the bones to be studied. The plan involved the determination of the ash content of each of the three major long bones of the right leg and of the combined bones of the left leg (1) with the epiphyseal cartilages retained and (2) with the epiphyseal cartilages removed.

To obtain supplementary information as a check on the ash content of the bones, it was planned to determine the calcium and phosphorus content of the blood serum and of the ash of the tibiae, and to note, by means of X-ray shadowgraphs, the age at which calcification occurred at the distal and proximal ends of the tibiae and the proximal end of the metatarsus. The blood-serum studies were conducted for 25 weeks; the other studies for 20 weeks.

EXPERIMENTAL MATERIAL AND METHODS

Three hundred and fifty chicks of each of the two breeds used (Rhode Island Red and Single-Comb White Leghorn) were hatched on April 8, 1931, at the United States Animal Husbandry Experiment Farm, Beltsville, Md., and were placed, the next day, in colony brooder houses on a grass range. At that time 100 chicks of each breed were selected at random and wing-banded. These birds were weighed then and at intervals of 2 weeks thereafter for 20 weeks, in order to obtain a reliable indication as to whether the average growth of the birds was satisfactory.

For the first 14 weeks, the birds were fed the following dry mash:

	Percent
Yellow corn meal	41.50
Ground wheat	20.00
Wheat bran	15.00
Dried buttermilk	10.00
Meat scrap (50 percent)	10.00
Calcite	2.75
Salt	.75
Total	100.00

In addition to this mash, during the first 6 weeks after hatching the birds were given all the freshly soured skim milk they would consume. After the fourteenth week and until the end of the twentieth week, all except 2.5 percent each of the meat scrap and dried buttermilk was replaced by yellow corn meal.

On the day after the chicks were hatched and at intervals of 1 week thereafter, a sufficient number of individuals of each breed was selected at random from among the unbanded birds to furnish the leg bones necessary for the ash determinations. The number for each sample varied from 1 to 15, depending on the size of the chicks.

Calcium and inorganic phosphorus determinations were made on the blood serum of the birds selected, in order that it might be known whether they were normal in this respect. Blood samples of approximately equal size were obtained by heart puncture from each of these birds before they were killed for bone samples. The blood

samples of birds of the same sex and breed were then combined for analysis. It was not possible, however, to differentiate readily between the sexes until the age of 6 weeks in the case of the Leghorns, and until the age of 7 weeks in the case of the Rhode Island Reds. Accordingly, until the sex of the chicks could be determined, the blood samples were combined with regard to breed only. The serum, obtained by centrifuging the blood samples, was analyzed for calcium by the Clark and Collip (*2*) [2] modification of the method of Kramer and Tisdall; and for inorganic phosphorus, by the method of Fiske and Subbarow (*3*).

After the collection of the blood samples, the birds were killed by cutting the jugular vein and puncturing the brain. Immediately after they were killed, X-ray shadowgraphs were made of the legs in order that the calcification of the bones might be studied by this means. The leg bones were then removed and dissected free of flesh in preparation for ashing. The epiphyseal cartilages were removed from the bones of one-half of the birds but not from the bones of the other half. Thus, one-half of the samples were made up of the diaphysis alone, and the other half included the diaphysis and the epiphyses. The long bones (femur, tibia with fibula, and metatarsus) of the left leg were combined in one sample, whereas the femur, tibia without fibula, and metatarsus of the right leg were studied separately.

During the first 6 weeks, the following numbers of birds were used in preparing each sample at the ages indicated.

Number of birds represented in each sample:	*Age*
15	1 day.
12	1 week.
8	2 weeks.
7	3 weeks.
6	4 weeks.
6	5 weeks.
5	6 weeks.

After the age of 6 weeks, one bird was sufficient for the weekly sample.

Bone samples of chicks of each sex were obtained as soon as the males could be readily distinguished from the females. As each bone was freed of flesh and its periosteum, it was placed in 95-percent ethyl alcohol and kept there until the determination of its ash content was begun. The following method of obtaining the ash content of the bones was used: The bones were removed from the alcohol, cut into small pieces with bone snips if they were inconveniently large, wrapped in filter paper, and extracted for 6 hours with 95-percent ethyl alcohol in a Soxhlet extractor. They were next allowed to dry in the open air and then extracted with diethyl ether for 6 hours. After being dried again in the air, the bones were placed in weighed crucibles, dried in a vacuum oven for 4 hours at a temperature of 60° C., and weighed. The weighed, oven-dried samples were then ashed to a constant weight, at 600°, to obtain the weight of ash in the moisture- and fat-free bones.

Previous, but unpublished, work at the United States Animal Husbandry Experiment Farm has shown that this method is efficient from the standpoint of accuracy and rapidity of determination. The

[2] Reference is made by number (italic) to Literature Cited, p. 1008.

extraction should be continued only long enough to insure removal of the soluble material. For this purpose, the writers have found 6 hours with each solvent to be sufficient, for the percentage of ash after such treatment was fully as high as that reported by St. John and his coworkers (*9*) when much longer periods were employed.

The amount of calcium and phosphorus in the ash of the tibiae was then obtained.[3] For this purpose, the ash was dissolved in dilute hydrochloric acid and the resulting solution was made up to a definite volume. Aliquot parts of this solution were used for the determination of calcium according to Kramer and Howland's (*8*) method for bone, and for the determination of phosphorus by the gravimetric method of the Association of Official Agricultural Chemists (*1*) for fertilizers.

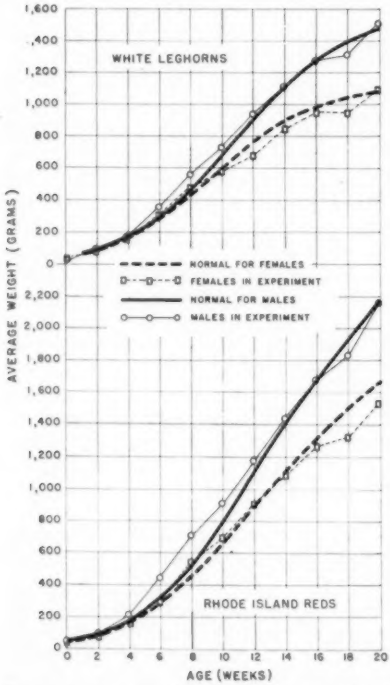

FIGURE 1.—Average weights, at 2-week intervals, of males and females of the two breeds compared with normal weights reported for these breeds by Hendricks, Lee, and Titus (*5*) and by Titus and Jull (*10*).

EXPERIMENTAL RESULTS

GROWTH OF THE BIRDS

The average growth (as measured by weight in grams) of the birds which were banded is shown in figure 1. There were 35 males and 44 females of the White Leghorn breed and 37 males and 45 females of the Rhode Island Red breed. The curves show that the growth was satisfactory as compared with the growth previously obtained at the Beltsville experiment farm with these breeds (*5*, *10*).

CALCIUM AND INORGANIC PHOSPHORUS CONTENTS OF BLOOD SERUM

The calcium and inorganic phosphorus contents of the blood serum of the birds at intervals of 1 week are shown in figure 2. All the values obtained were well within the normal limits, as shown by unpublished results; in fact, there was less variation than is frequently observed under experimental conditions. The variation was so small throughout most of the first 20 weeks as to have no apparent significance. Shortly after the reduction of the relative quantities of the protein concentrates in the diet of the birds at the age of 14 weeks, there was a decrease for about 4 weeks in the inorganic phosphorus content of the blood serum, whereas the calcium content showed

[3] The determinations of the calcium and phosphorus in the ash of the tibiae were carried out by R. E. Davis and G. H. Kennedy, of the Animal Husbandry Division.

no change. This decrease in phosphorus content was then followed by an increase for 2 or 3 weeks and then by another decrease. There was, apparently, no significant difference in the calcium and inorganic phosphorus contents of the blood serum of the males and females from the sixth or seventh week, when sex was first differentiated, to the twentieth week.

Determinations of the calcium and inorganic phosphorus in the blood serum of the birds were continued from the ages of 20 to 25 weeks, with the special object of observing the changes in the calcium of the blood serum of the pullets as they began to lay, as compared with those changes in the blood serum of the males of the same ages. Figure 2 shows a very marked increase in the case of the White Leghorn pullets after the twentieth week and a similar increase in the case of the Rhode Island Red pullets after the twenty-third week. This increase was undoubtedly due to the increased demand for calcium to be used in egg production, as the pullets began to lay at about this time. Halnan (*4*) has shown that there is an increased retention of calcium from the feed during the week preceding the beginning of egg production, and Hughes and his coworkers (*7*) have found that there is an increased content of calcium in the blood serum of pullets at the time that laying begins.

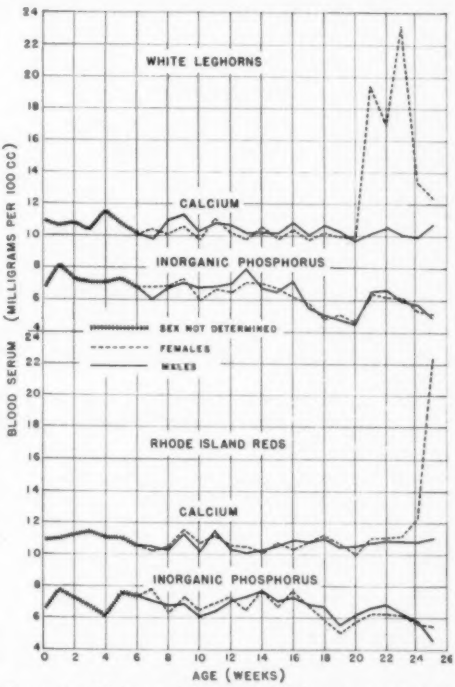

FIGURE 2.—Calcium and inorganic phosphorus contents of the blood serum of White Leghorns and Rhode Island Reds at weekly intervals.

CALCIFICATION OF EPIPHYSES

Through an examination by the X-ray shadowgraphs, certain stages in the calcification of the epiphyses were noted. During the first week of the life of the chick, centers of ossification appeared in the cartilages at the distal end of the tibia and the proximal end of the metatarsus. There was no difference between the two breeds in this respect. Centers of ossification appeared in the cartilage at the proximal end of the tibia by the end of the sixth week. In this respect also no difference was noted between the two breeds.

After the first appearance of the centers of ossification the areas undergoing calcification gradually increased in size until they made up most of the epiphyses and the epiphyses became attached to the

diaphysis. The union of a diaphysis with an epiphysis occurred first at the proximal end of the metatarsus, then at the distal end of the tibia, and later at the proximal end of the tibia. There seemed to be a tendency for the epiphyses of the females to calcify at an earlier age than those of the males, and for those of the White Leghorn birds to calcify at an earlier age than those of the Rhode Island Reds (table 1).

TABLE 1.—*Effect of breed and sex on the age at which the calcification of the epiphyses were apparently complete, as shown by the X-ray shadowgraphs*

Breed and sex of birds	Age of bird when calcification occurred at—		
	Proximal end of metatarsus	Distal end of tibia	Proximal end of tibia
White Leghorn:	*Weeks*	*Weeks*	*Weeks*
Female	12	13	19
Male	14	15	20
Rhode Island Red:			
Female	13	17	20
Male	14	19	(a)

a Not complete at twentieth week.

ASH CONTENT OF BONES

The percentage of ash in the moisture- and fat-free bones each week of the experiment is presented in figures 3 and 4, and the percentages

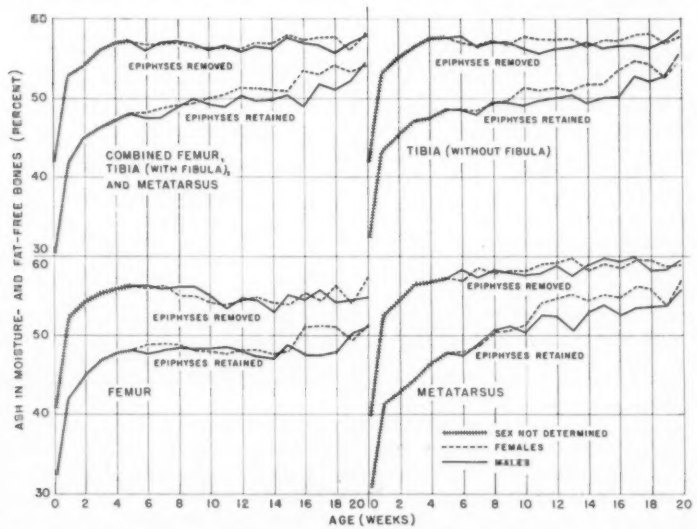

FIGURE 3.—Ash content of the bone samples obtained from the White Leghorns at weekly intervals.

at 4, 5, 6, 7, 8, and 20 weeks are given in table 2. The curves are comparatively smooth during the first few weeks, when the determinations of ash were made on samples composed of bones of from 5 to 15 individuals. Later, when the samples represented single birds, these curves are not so smooth.

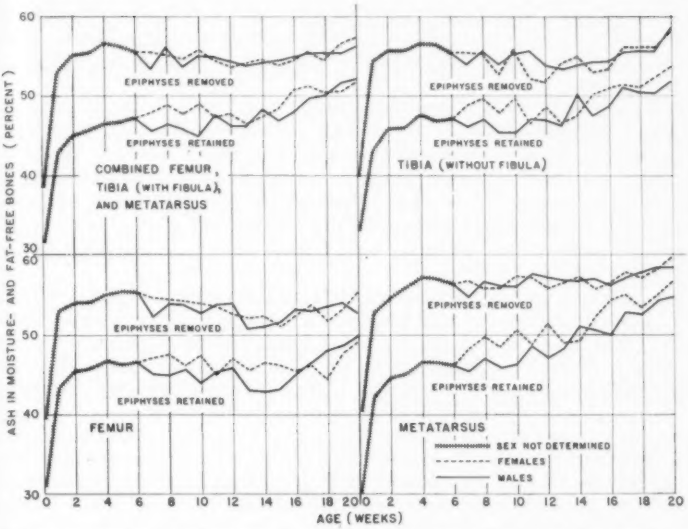

FIGURE 4.—Ash content of the bone samples obtained from the Rhode Island Reds at weekly intervals

TABLE 2.—*Percentage of ash in the moisture- and fat-free bones of the White Leghorns and the Rhode Island Reds at the ages indicated*

WHITE LEGHORNS

Age in weeks	Sex	Bones with cartilage retained				Bones with cartilage removed			
		Femur	Tibia	Metatarsus	Combined bones	Femur	Tibia	Metatarsus	Combined bones
		Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
4	Not separated	47.8	47.5	46.4	47.3	55.9	57.4	56.7	56.9
5	do	48.2	48.4	47.7	48.1	56.1	57.6	57.2	57.1
6	Male	47.8	48.7	47.6	47.6	56.3	57.8	58.4	56.0
	Female	48.8	48.6	47.6	48.1	56.0	57.1	56.8	56.9
7	Male	48.2	48.1	49.0	47.6	56.0	56.4	57.3	57.1
	Female	48.8	48.4	48.6	48.8	56.2	56.7	58.4	56.8
8	Male	48.5	49.5	50.8	49.6	56.0	57.0	58.3	57.2
	Female	48.7	49.4	50.5	49.0	55.1	57.3	57.9	56.9
20	Male	50.9	55.8	55.9	54.4	54.8	59.6	59.4	57.9
	Female	51.2	54.6	56.8	54.3	57.3	57.7	58.8	58.2

RHODE ISLAND REDS

4	Not separated	46.7	47.6	46.5	46.5	55.0	56.6	57.0	56.7
5	do	46.3	46.8	46.5	46.6	55.3	56.4	56.9	56.5
6	do	46.4	47.0	46.1	47.3	55.3	55.4	56.3	55.6
7	Male	45.0	46.1	45.5	45.9	52.2	54.0	54.6	53.5
	Female	47.1	49.0	48.3	48.0	54.6	55.4	56.8	55.5
8	Male	45.0	47.0	46.9	46.6	53.7	55.8	56.6	56.1
	Female	47.4	49.8	49.7	49.1	54.4	55.4	55.8	55.1
20	Male	50.0	51.8	54.4	52.2	52.6	58.5	58.3	56.0
	Female	49.0	53.6	56.6	51.8	55.3	58.5	59.8	57.4

In general, the ash content of the moisture- and fat-free bones of the chicks at the age of 1 day was approximately 40 percent when the epiphyseal cartilages were removed and 30 percent when they were

retained. There was a marked increase in the ash content during the first week, and this increase continued, but at a progressively slower rate, during the next few weeks. Thereafter, there was relatively little change.

A comparison of the curves representing the ash content of the bones, with and without the epiphyseal cartilages, shows that the diaphysis contains an appreciably higher percentage of ash than does the diaphysis with the epiphyses. This difference is greatest during the first few weeks, after which the curves representing the percentage of ash in the bones prepared in the two different ways tend to approach each other gradually and are fairly close together at the end of 20 weeks.

The ash content of the bones obtained from the White Leghorns was higher than that of the corresponding bones of the Rhode Island Reds. This difference continued throughout most of the period of 20 weeks covered in the present study. After approximately the nineteenth week, however, there was a tendency for the ash content of the bones of the Rhode Island Reds to become very nearly equal to that of the bones of the White Leghorns. Thus it appears that calcification of the leg bones proceeds at a higher rate and is completed at an earlier age in the White Leghorns than in the Rhode Island Reds. Such a difference is to be expected, since the former matures somewhat more rapidly than the latter.

Another point brought out by these curves is that throughout most of the period when the males and females were studied separately the average ash content of the leg bones of the females from which the cartilage had not been removed was higher than that of the leg bones of the males. However, at the twentieth week there was, in general, very little difference. In contrast to this, the ash content of the bones from which the cartilage had been removed was practically the same for the two sexes.

A comparison of the three long bones of the leg shows that the metatarsus had the most ash, the femur the least, and the tibia an intermediate amount. During the preparation of the samples a corresponding difference was noted in the physical character of these three bones. The metatarsus was yellowish white in color, had a yellow marrow, greater strength, and less brittleness than either of the other two bones. There was less difference between the femur and the tibia. Both of them were grayish white, had a red marrow, and tended to shatter readily when cut with the bone snips, in contrast to the metatarsus which permitted the bone snips to cut through cleanly. The femur, however, seemed to be somewhat more brittle than the tibia. Thus, it appears that the ash content of the bones bears some relation to their physical character.

CALCIUM AND PHOSPHORUS CONTENTS OF THE ASH OF THE TIBIA

There was relatively little change in the calcium and phosphorus contents of the ash of the tibiae throughout the 20 weeks and, for this reason, only the averages of the results obtained are given in table 3.

After the first week the values ranged between approximately 34.5 and 37 percent of calcium and 17.5 and 18.5 percent of phosphorus. There was apparently no difference in this respect between the bones of cockerels and pullets or between birds of different ages.

TABLE 3.—*Effect of age and sex on average percentage of calcium and phosphorus in the ash of the tibiae of White Leghorns and Rhode Island Reds*

WHITE LEGHORNS

Age of birds	Sex of birds	Bones with cartilage retained			Bones with cartilage removed		
		Ca	P	Ca/P ratio	Ca	P	Ca/P ratio
		Percent	*Percent*		*Percent*	*Percent*	
1 day	Not separated	31.3	17.7	1.77:1	35.0	20.4	1.72:1
1–5 weeks	do	35.0	18.1	1.94:1	34.2	17.8	1.94:1
6–20 weeks	Male	35.7	17.9	1.98:1	36.7	18.2	2.02:1
	Female	35.9	17.6	2.05:1	36.2	18.1	2.00:1

RHODE ISLAND REDS

1 day	Not separated	33.4	18.9	1.77:1	35.0	19.8	1.77:1
1–6 weeks	do	34.5	17.9	1.93:1	34.8	17.9	1.96:1
7–20 weeks	Male	35.3	17.8	1.97:1	36.1	18.1	1.99:1
	Female	36.7	18.4	2.00:1	36.7	18.4	2.00:1

The ratio of calcium to phosphorus in the ash of the tibiae was found to be very close to 2.0:1 in most of the samples; therefore the averages of the values, given in table 3 for the different breeds and sexes, in most instances are also very close to 2.0:1. However, one exception to the foregoing statement is that of the calcium-phosphorus ratio of the ash of the tibiae in birds aged 1 day. The average ratio was 1.77:1 in three cases and 1.72:1 in the fourth.

DISCUSSION

The data presented in this paper on the ash content of the moisture- and fat-free leg bones of chickens indicate that there was little tendency for the percentage of ash in those bones from which the cartilage had been removed to change after the fourth or fifth week. There appears, however, to have been a slight tendency for the ash content of the metatarsus to increase with the age of the chick and for that of the femur to decrease between the fifth and fifteenth week. Nevertheless, in both cases the maximum deviation from the average ash content observed between the seventh and twentieth week was less than 4 percent of the average.

Although the data show that the ash content of the bones from which the epiphyseal cartilages had been removed tended to remain rather constant from the fourth to the twentieth week, they also show that there was a gradual increase, during this period, in the ash content of the bones (other than the femur) from which the cartilages had not been removed. It appears, therefore, that this change was due to a progressive calcification of the epiphyseal cartilages. In any case, the ash content of the bones from which the cartilages had not been removed tended to become more nearly equal to that of the bones from which the cartilages had been removed as the birds approached maturity and the calcification of the epiphyses became more nearly complete.

The tendency for the ash content of the diaphysis to remain rather constant under normal conditions does not necessarily mean that the ash content may not change under certain conditions, for unpublished

experiments by the writers have shown that it may change either as the result of the occurrence of rickets or because of a change in the rate of growth. If the rate of growth is slow, the cartilage-free bones may have an abnormally high ash content. On the other hand, very rapid growth, especially after a period of slow growth, tends to bring about a decrease in the ash content of both the diaphysis and the epiphyseal cartilage. It is quite probable that much of the variation among individual chickens of the ash content of their leg bones is due largely to differences in their rate of growth.

It is necessary, therefore, to consider the rate of growth of the chickens in any experimental or regulatory work with feeds in order that errors in the interpretation of results may not occur. Thus it is possible that a diet containing a cod-liver oil low in vitamin A might contain an inadequate supply of this vitamin. The chickens receiving such a diet would tend to grow more slowly than others receiving an adequate supply, and probably the percentage of ash in their bones would be higher than if their growth had been normal. Failure to take the growth rate into account would lead to the conclusion that the antirachitic value of the oil under consideration was higher than was actually the case.

The results of the present study indicate that, in most cases, when the birds are between the ages of 7 and 20 weeks, the ash content of the females' bones with the cartilage retained is greater than that of the males' bones. The X-ray shadowgraphs furnish evidence that this difference is due to an earlier calcification of the epiphyses of the bones of the females rather than to a greater storage of minerals in preparation for egg production, as was suggested by Holmes, Pigott, and Moore (*6*). Furthermore, the bones of the males, either with or without their cartilages, contain approximately the same proportion of ash at the age of 20 weeks as do those of the females. Moreover the greater weight of the bones of the males, as is evident from their greater body weight, indicates a larger total quantity of ash stored in their skeletons.

Since the above-mentioned difference was found to be due to an earlier calcification of the cartilages in the case of the females, it can be avoided in experimental studies by removing the epiphyseal cartilages from all bones to be ashed. Furthermore, if the epiphyseal cartilages are removed in preparing the samples, the writers' results indicate that it is unnecessary to consider the sex of the individual or its age, between the fifth or sixth and the twentieth week. The removal of the cartilages in the preparation of the bone for ashing makes the dissection of the bone easier and more accurate, for the cartilages can be removed very readily with the flesh and periosteum. The accuracy of the dissection is an important point, for the writers have found, in unpublished work, that there is more danger of error resulting from faulty dissection than from any other step in the analytical technic.

The objection may be raised that the diaphysis alone would not indicate the extent of the occurrence of rickets so well as would the diaphysis together with the epiphyseal cartilages. St. John, Kempf, and Bond (*9*) found that between normal and rachitic chicks there was a greater difference in the ash content of the epiphyseal cartilages than in that of the diaphyses. Although this is true, it is also true

that after the first few weeks the epiphyses make up only a small portion of the combined weight of the bone and cartilages and would, therefore, have less influence on the ash content of the sample than would the diaphysis. The writers have found, in unpublished data, that the ash content of the diaphyses of bones obtained from rachitic chicks is almost as low as the ash content of the diaphysis and epiphyses combined. They conclude, therefore, that it is entirely satisfactory to use the percentage of ash in the diaphyses of the leg bones as a measure of the extent of the development of rickets in the chicken.

The data obtained by the writers seem to indicate that the tibia would ordinarily be most satisfactory for determining the ash content of the leg bones of chickens reared under normal conditions. The ash content of the tibia agrees closely with that of the combined long bones of the leg, and the use of the tibia alone has the advantage of simplifying the analytical procedure. This bone has also been generally used in the work reported in the literature.

SUMMARY AND CONCLUSIONS

Two groups of chickens, one consisting of 350 Single-Comb White Leghorns and the other of 350 Rhode Island Reds, were reared on a grass range under normal conditions. The ash content of the moisture- and fat-free leg bones of representative individuals from each group was determined each week during the first 20 weeks of their lives. Stages of calcification of the epiphyses in the leg bones of these birds were noted by means of X-ray shadowgraphs. The contents of the calcium and inorganic phosphorus of the blood serum and of the ash of the tibiae also were obtained.

There was no significant change in the calcium content of the blood serum of the birds with increasing age, except for a marked increase in the case of the pullets just before they commenced to lay. After the fourteenth week there were marked changes in the inorganic phosphorus content of the blood serum, which were probably due in part to increasing age and in part to a change in the diet which was made at that time.

The X-ray shadowgraphs indicated an earlier calcification of the epiphyses of the bones of the females than of the males and also an earlier calcification of the epiphyses of the bones of the White Leghorns than of the Rhode Island Reds.

There were no significant differences in the calcium and phosphorus contents of the ash of the tibiae, nor was the difference significant between the sexes. The ratio of calcium to phosphorus in the ash of the tibiae was noticeably low on the day after hatching, after which it increased to approximately 2.0:1 in all cases.

The ash content of the diaphysis of the long bones of the legs of chickens of both sexes tended to remain constant between the fifth and twentieth weeks. In general, the ash content of the diaphysis with its cartilages was higher in the case of the females than in the case of the males between the sixth or seventh and the eighteenth week, depending on the breed.

It is concluded that the tibia is the most satisfactory bone to use in studying the effect of various diets and sources of vitamin D on the ash content of the bones of chickens. It is recommended that the epiphyseal cartilages, together with the periosteum, be removed in the preparation of the bone for ashing.

LITERATURE CITED

(1) Association of Official Agricultural Chemists.
1930. official and tentative methods of analysis. Ed. 3, 593 pp., illus. Washington, D.C.
(2) Clark, E. P., and Collip, J. B.
1925. a study of the tisdall method for the determination of serum calcium with a suggested modification. Jour. Biol. Chem. 63: 461–464.
(3) Fiske, C. H., and Subbarow, Y.
1925. the colorimetric determination of phosphorus. Jour. Biol. Chem. 66: 375–400.
(4) Halnan, E. T.
1925. the calcium, phosphorus and nitrogen balance of the non-laying pullet. Jour. Natl. Poultry Inst. 10: 410–416.
(5) Hendricks, W. A., Lee, A. R., and Titus, H. W.
1929. early growth of white leghorns. Poultry Sci. 8: 315–327.
(6) Holmes, A. D., Pigott, M. G., and Moore, W. B.
1932. the influence of sex on the size and composition of the tibiae of growing chicks. Poultry Sci. 11: 243–249, illus.
(7) Hughes, J. S., Titus, R. W., and Smits, B. L.
1927. the increase in the calcium of hen's blood accompanying egg production. Science (n.s.) 65: 264.
(8) Kramer, B., and Howland, J.
1926. the quantitative estimation of calcium, magnesium, phosphate, and carbonate in bone. Jour. Biol. Chem. 68: 711–719.
(9) St. John, J. L., Kempf, C., and Bond, L.
1933. observations on the bone ash method of determining the effectiveness of vitamin d supplements. Poultry Sci. 12: 34–36.
(10) Titus, H. W., and Jull, M. A.
1928. the growth of rhode island reds and the effect of feeding skim milk on the constants of their growth curves. Jour. Agr. Research 36: 515–540, illus.

THE LETHAL EFFECT OF LOW TEMPERATURES ON THE VARIOUS STAGES OF THE CONFUSED FLOUR BEETLE [1]

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INTRODUCTION

Entomologists know that stored-product insects are not very resistant to low temperatures. Refrigeration is employed commercially to prevent insect damage. Ordinarily, insect activity is suspended in infested articles, or the infestation by insects is prevented in "sterilized" goods. However, low temperature is rarely used as the sterilizing agent in place of heat or a fumigant.

As compared with the attention paid to the effect of high temperature, but little investigation has been made of the lethal effect of low temperatures. It must be recognized that the cold resistance of different species of insects may vary widely, whereas the heat resistance varies only slightly. In order to obtain the most economical combination of the temperature and the length of exposure, actual data are necessary regarding the time required to kill a certain insect species at any given low temperatures.

Because the confused flour beetle (*Tribolium confusum* Duval) is an insect with which prepared food products are likely to become infested, it was selected for the study reported in this paper. The data presented are of particular interest also for their physiological significance.

In applying time-temperature data to the effect of low temperatures on insects, it should be understood that allowance must be made for the time necessary to cool the insect-containing medium, as, for example, wheat flour. The measurement of the cooling (heat transfer) time of standard volumes of various commercial products is a separate study which will not be considered here.

HISTORICAL REVIEW

Most of the data available in the literature regarding the lethal effects of low temperatures on stored-product insects are merely isolated observations, often presented without the time of exposure being reported. The first accurate measurement of the effect of refrigeration on an insect was made by Back and Pemberton (*1*, *2*),[2] who found it possible to kill the early stages of the Mediterranean fruit fly by cold storage.

Although some data published relative to this subject are available for other stored-product insects, only one reference to the effect of low temperatures on *Tribolium confusum* is known. Payne (*8*) says that normally it may recover from temperatures as low as $-14°$ C., but she makes no mention of the duration of the exposure.

[1] Received for publication Jan. 4, 1934; issued July 1934. Paper no. 1235 of the Scientific Journal Series of the Minnesota Agricultural Experiment Station.

[2] Reference is made by number (italic) to Literature Cited, p. 1016.

EXPERIMENTAL METHOD

A Carrier air-conditioned cabinet was employed in the experiments involving temperatures from 0° to −18° C. A variation of less than 1° was obtained for the period of each experiment. For the experiments in which higher temperatures were used another type of cabinet was utilized in which the temperature varied only slightly over a period of several weeks.

Tribolium confusum were cultured in fine whole-wheat flour, about 500 grams for each lot. The flour was sifted through a standard no. 6 silk bolting cloth (74 meshes to the linear inch). About 3,000 adult beetles were placed in each lot. The cultures were covered with muslin and kept at a constant temperature of 27° C. Eggs were seldom allowed to accumulate more than 24 hours.

All stages except the adults were exposed in small glass vials, 50 individuals in each, with a few exceptions when 100 were so exposed. The eggs were counted under a binocular microscope, only those which were normally plump being used. The unstoppered vials with their contents were placed in the temperature cabinets. After exposure each vial was lightly stoppered with cotton or with cork stoppers having screen-covered holes. Adults were exposed in silk bolting-cloth cylinders. This precaution was necessary because preliminary tests had shown that the excited adults give off an odoriferous vapor. When confined with this gas they soon die, 95 percent being killed in 4 or 5 hours.

During the earlier experiments exposed eggs without flour were placed in a cabinet and kept at a temperature of 27° C. During the hatching, results were noted daily, the young larvae being removed from the vials and discarded. Later tests showed that the results were the same if eggs were allowed to incubate in about 2 grams of flour and the larvae counted 3 or 4 days after hatching began. The tedious work of noting results was thus greatly reduced. All other stages of the insect were treated in this manner. Check experiments of 50 individuals each were always included in each series. They were kept at 27° and their condition noted only after the observations on the exposed insects had been completed.

All experiments at 7° C. were carried out in glass desiccators at three different relative humidities, viz. 6.2 (7-mm saturation deficiency), 50, and 73.7 percent. With the exception of five experiments which were made without moisture control, all experiments at 12° were carried out likewise in desiccators at relative humidities of 32.8 (7-mm saturation deficiency), 50, and 73.7 percent. At both temperatures no differences in the lethal effects at different humidities were observed, neither did there appear to be any difference in the results obtained from the experiments conducted outside the desiccators. Accordingly, all the experiments at each of these temperatures were grouped without regard to moisture conditions.

The percentage of mortality in each case was calculated by Abbott's formula, $\frac{x-y}{x}(100)$, where x is the percentage survival in the check and y that in the exposed lot. In figure 1 are shown curves for eggs of three age groups at 12° C. Fifty and 100 percent mortalities were estimated in all cases from curves of this type. Each point in the curve shown is an average of the results of from 1 to 14 experiments,

each involving 50 individual insects. Although a few points are based on a single experiment, no curve is drawn through less than eight points.

INFLUENCE OF AGE ON COLD RESISTANCE OF EGGS

Davis (7), studying the effects of high and low temperatures upon the mosquito *Aedes aegypti* Linn., found that at −5.5° C., freshly laid eggs were slightly more resistant than mature eggs. The writers have found the same thing to be true for eggs of *Tribolium confusum* that have been exposed to chloropicrin.

In order to study the effect of age on the resistance of eggs of *Tribolium confusum* to low temperature, eggs were placed in six age groups, namely, 1 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, and 120 to 132 hours old. At 27° C., the temperature at which the sifted eggs were stored, those 132 hours old were nearly ready to hatch. Eggs in the different age groups were simultaneously exposed to low

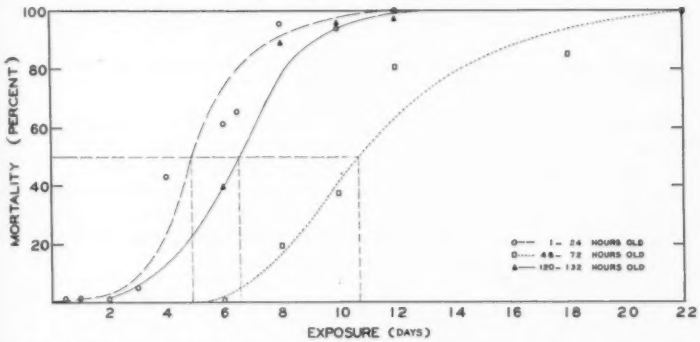

FIGURE 1.—Mortality of eggs of *Tribolium confusum* of different ages when exposed to a temperature of 12° C.

temperatures and otherwise treated as nearly alike as possible. In counting the survivors of these tests, only those larvae which previous experience showed to be able to complete their development were counted. Certain larvae died after emerging partly from the egg. Others hatched in such a weakened condition that they also soon died. All such larvae were considered as having been killed in the egg stage. The eggs set aside as checks showed from 78.38 to 90 percent survival as calculated from the number of young larvae hatched from them.

Figure 1 shows the average results at each age-temperature combination. In table 1 is given a summary of the time required at the various temperatures to obtain 50 and 100 percent mortality. The latter figures do not possess, for comparative purposes, the precision of the previous ones, but they are more useful in estimating time-temperature relations for insect control. Although eggs were separated into 6 age groups most of the experiments were confined to 3 ages, namely, 1 to 24, 48 to 72, and 120 to 132 hours old. A few results with eggs at other ages are given for the sake of showing the influence of the entire range of age on the lethal effects of temperature at −12° C.

TABLE 1.—*Effect of low temperatures on eggs of Tribolium confusum of different ages, 50 and 100 percent mortality being the basis of comparison*

50 PERCENT MORTALITY

Temperature (° C.)	Number of hours of exposure required to produce mortality in eggs of—					
	Age 1 to 24 hours	Age 24 to 48 hours	Age 48 to 72 hours	Age 72 to 96 hours	Age 96 to 120 hours	Age 120 to 132 hours
12	118		258			157
7	43		214			110
0	4.75	4.5		11.25		
−4	1.5	1.5	3.5	3.75	2.75	
−6	.75		3.25			4
−12	.75	1.5	3.5	6	2.75	1.5
−12 [a]	.75		.75			1.25
−18	.25		.25			.25

100 PERCENT MORTALITY

12	288		528			312
7	216		432			288
0	13	11.5		16	13.5	
−4	9.25	8.25	7.5	9.5	8.25	
−6	6.5		8			13
−12	9.5	9.5	14	12	9.5	6
−18	1		1			1

[a] The second series of experiments at −12° C. included fewer eggs for the determinations at 1 to 24 hours and at 48 to 72 hours than in the first series at that temperature.

The results show that in general the resistance of the eggs is probably greatest in the fourth age group which includes eggs 72 to 96 hours old. According to available embryological data for *Tribolium confusum* at this age, the embryos have just undergone dorsal closure and the young larvae are becoming rather well formed. It is known for insect embryos in general that their respiration is at a lower level here than at other stages of their development.

INFLUENCE OF AGE ON COLD RESISTANCE OF LARVAE

For the purposes of the experiments with larvae of *Tribolium confusum* three age groups were selected. The first group consisted of unfed larvae 1 to 12 hours old, averaging 6 hours. The second and third groups of about half-grown and of full-grown larvae, respectively, were raised on whole-wheat flour, from eggs the age of which was known for each lot. Because larvae of the same age varied in size considerably under the culture conditions, a few third- and sixth-instar larvae were selected by the aid of the measurements given by Chapman (*5*) and by Brindley (*3*). Then by comparison it was possible to select with a fair degree of accuracy the larvae of these instars from the cultures in which they predominated. Only active larvae able to resist jarring from a sheet of paper were used, thus eliminating those in the process of molting.

The manner in which the larvae were handled was practically the same as that for the eggs. Fifty individuals were used in each experiment. Observations were made regularly 1 week after treatment. The groups of larvae set aside as checks showed 91.2 to 100 percent survival.

In table 2 is summarized the time in hours required to obtain 50 and 100 percent mortality for the three age groups of larvae at different low temperatures. These data indicate that the full-grown larvae are most resistant, although the difference is pronounced only at 7° C.

Larvae killed outright by freezing invariably turn black soon afterward. Some others turn black in irregular areas. Many larvae which otherwise appeared normal were unable to void feces properly. In some cases long, sticklike strands protruded from the anus a distance equivalent to the length of the entire body. In full-grown larvae certain time-temperature combinations produced rare winged freaks, examples of metathetely.[3]

TABLE 2.—*Effect of low temperatures on larvae of Tribolium confusum at different stages of growth, 50 and 100 percent mortality being the basis of comparison*

Temperature (° C.)	Number of hours of exposure required to produce mortality in larvae of—		
	Age 1 to 12 hours	Third instar	Sixth instar
50 PERCENT MORTALITY			
7	134.4	149	197
−6	10	9	11.75
−12	.42	.35	.48
−18	.18	.17	.17
100 PERCENT MORTALITY			
7	288	250	528
−6	16	16	16
−12	1.75	.5	1
−18	1	.5	.5

EFFECT OF LOW TEMPERATURES ON PUPAE

Pupae of only one age were selected. In general, the lighter-colored ones are youngest, and these were chosen for use in the tests, Each lot of treated pupae was placed in about 2 grams of flour at 27° C. Mortality was based on the percentage emergence of the adults. The groups of pupae set aside as checks showed a high proportion of emergence, 96.32 to 100 percent.

In table 3 are summarized the times in hours required to obtain 50 and 100 percent mortality of pupae at different low temperatures. Results at 12° C. are not tabulated because exposures over a long time showed very little mortality. The keeping of 100 pupae at 12° for 22 days resulted in only 4 dead specimens. No adults emerged during this long exposure.

TABLE 3.—*Effect of low temperatures on pupae of Tribolium confusum*

Temperature (° C.)	Hours of exposure required to produce—	
	50 percent mortality	100 percent mortality
7	258	432
−6	.67	10
−12	.63	1.5
−18	.13	.5

[3] Chapman (*6, p. 294*) refers to Strickland's study of parasitized *Simulium* larvae in which Strickland found that the action of the parasite prevented the wing histoblasts from developing normally, the wing pads being greatly retarded and reduced. To this phenomenon, Strickland gave the term "metathetely" (to run behind completion).

EFFECT OF LOW TEMPERATURES ON ADULTS

Adults ranging in age from 1 to 5 months were used, no attempt being made to study the effect of age on their resistance to low temperatures. As the adults live a year or more, those used were young and vigorous. Treated adults were kept under observation for a week or so without flour. Check groups of adults under these conditions showed 94.66 to 96 percent survival.

In table 4 are summarized the times in hours required to obtain 50 and 100 percent mortality of adults at different low temperatures. Exposures of adults at 12° C., as in the case of pupae at this temperature, are not tabulated. Some activity and little mortality of adults occurs at this temperature, groups being kept at different humidities for from 22 to 35 days without showing over 6 percent mortality.

TABLE 4.—*Effect of low temperatures on adults of Tribolium confusum*

Tempera- (° C.)	Hours of exposure required to produce—	
	50 percent mortality	100 percent mortality
7	336	528
− 6	8.4	24
−12	.23	2
−18	.15	.5

As mentioned before, it was necessary to place the adults for treatment in bolting-cloth cylinders to prevent accumulation of the toxic vapors given off by the excited beetles. Adults injured by low temperature also give off this vapor which, if the beetles are placed in flour, imparts a definitely pink color to that flour. Several series of experiments indicated that the intensity of this color varied directly with the length of exposure to low temperature. Chapman (*6*) noted this color in flour and other materials, stating that the vapor is given off by beetles irritated mechanically.

Adults not injured sufficiently to die as a result of their cold treatment were counted as survivors. It was found that survivors could usually be easily determined after a week was allowed for recovery. In two instances adults exposed to −12° C. for 45 minutes were still alive after 45 days, but they were never able to crawl about. Inability to void feces properly was common among injured adults as in the case of the larvae. Whenever the recovery of an individual was in doubt, it was counted as a survivor. Some living adults, although more or less paralyzed after they had been exposed to −12° for 45 minutes, produced fertile eggs. Survivors of 6 hours exposure to −6° produced many fertile eggs. Four hundred adults were kept in 300 grams of flour at 12° for 29 days, but no eggs were laid at that temperature. Several days after the removal of these adults from the low temperature 400 to 500 eggs were recovered, indicating that egg laying was normal after such a period of inactivity.

RELATIVE RESISTANCE OF DIFFERENT STAGES

Table 5 shows the time-temperature dosages required to kill both 50 and 100 percent of the different stages of the confused flour beetle. In the case of the eggs and the larvae, several ages of which were exposed to low temperatures, the results for the most resistant ages are given.

TABLE 5.—*Effect of low temperatures on the various stages of Tribolium confusum 50 and 100 percent mortality being the basis of comparison*

50 PERCENT MORTALITY

Stage	Number of hours of exposure required to produce mortality at temperature of—				
	7° C.	0° C.	−6° C.	−12° C.	−18° C.
Egg	214	11.25	4	3.5	0.25
Larva	197		11.75	.48	.18
Pupa	258		.67	.63	.13
Adult	336		8.4	.23	.15

100 PERCENT MORTALITY

Egg	432	16	14	14	1
Larva	528		16	1.75	1
Pupa	432		10	1.5	.5
Adult	528		24	2	.5

It is apparent from the figures for 50 percent mortality that the adults are more resistant than the other stages at the moderately low temperature of 7° C. At −6°, however, the larvae are about as hard to kill as the adults and at −12° the eggs are by far the most resistant. The pupae succumb more readily than the other stages.

The figures for 100 percent mortality provide answers to the practical questions of how low a temperature and how long an exposure is necessary to kill all stages of the confused flour beetle. It is possible to kill every stage of the insect at 7° C. (44.6° F.) if held at that temperature for nearly 25 days (600 hours). Only 24 hours' exposure is necessary if the temperature can be lowered to a little below −6° C. (21.2° F.).

DISCUSSION

In no previous instance apparently have the effects of low temperatures on insects been compared on the basis of 50 percent mortality. Carter (*4*) compared the resistance of various stages of the bean weevil (*Mylabris obtectus* Say) on the basis of 100-percent kill. All stages were killed only after several hours exposure to temperatures of −12° to −15° C., whereas *Tribolium confusum* in every stage but the egg succumbs in 2 hours or less at −12°. Robinson (*9*) determined the resistance of the rice and the granary weevils (*Sitophilus oryzae* L. and *S. granarius* L.) to various low temperatures at intervals from their respective thresholds of activity to as low as −17.7°. From a comparison of his data with those given here for *T. confusum* it appears that the latter species is nearly as susceptible to low temperatures as the rice weevil. Given the same low temperature in their environments, it is much easier to kill the confused flour beetle with cold than it is the granary weevil. Robinson reports that *S. granarius* requires 100 hours at −6.6° for a complete kill, whereas it has been shown here that all stages of *T. confusum* are killed at −6° in 24 hours.

SUMMARY

A study was made of the lethal effect of low temperatures on four stages of the confused flour beetle (*Tribolium confusum* Duval)—egg, larval, pupal, and adult. Data were obtained showing the number of

hours of exposure required to produce 50- and 100-percent mortality, the temperatures ranging from 12° to −18° C.

The eggs were placed in six groups according to age, 1 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, and 120 to 132 hours. Resistance to cold was found to be greatest in the fourth age group, 72 to 96 hours.

The larvae were placed in three age groups—unfed larvae 1 to 12 hours old, half-grown, and full-grown larvae. The full-grown larvae were found to be the most resistant, although the difference was pronounced only at 7° C.

The mortality of the pupae was based on the percentage of emergence of the adults.

The adults ranged in age from 1 to 5 months, and those not injured sufficiently to die as a result of exposure to low temperatures were counted as survivors.

The figures for 50-percent mortality show that the adults were more resistant than the other stages at 7° C.; at −6° the larvae were about as resistant as the adults; and at −12° the eggs were the most resistant.

The figures for 100-percent mortality show that all stages of the insect can be killed at 7° C. if exposed at that temperature for 25 days; but if the temperature is lowered to −6°, only 24 hours' exposure is necessary.

LITERATURE CITED

(1) Back, E. A., and Pemberton, C. E.
1916. effect of cold-storage temperatures upon the mediterranean fruit fly. Jour. Agr. Research 5: 657–666.

(2) ——— and Pemberton, C. E.
1916. effect of cold-storage temperatures upon the pupae of the mediterranean fruit fly. Jour. Agr. Research 6: 251–260.

(3) Brindley, T. A.
1930. the growth and development of ephestia kuehniella zeller (lepidoptera) and tribolium confusum duval (coleoptera) under controlled conditions of temperature and relative humidity. Ann. Ent. Soc. Amer. 23: 741–757, illus.

(4) Carter, W.
1925. the effect of low temperatures on bruchus obtectus say, an insect affecting seed. Jour. Agr. Research 31: 165–182, illus.

(5) Chapman, R. N.
1918. the confused flour beetle (tribolium confusum duval). Minn. State Ent. Rept. 17: 73–94, illus.

(6) ———
1926. inhibiting the process of metamorphosis in the confused flour beetle (tribolium confusum, duval). Jour. Expt. Zool. 45: 293–299.

(7) Davis, N. C.
1932. the effects of heat and cold upon aedes (stegomyia) aegypti. Amer. Jour. Hyg. 16: 177–191.

(8) Payne, N. M.
1927. two factors of heat energy involved in insect cold hardiness. Ecology 8: 194–196.

(9) Robinson, W.
1926. low temperature and moisture as factors in the ecology of the rice weevil, sitophilus oryza l., and the granary weevil, sitophilus granarius l. Minn. Agr. Expt. Sta. Tech. Bull. 41, 43 pp., illus.

INCREASE OF KERNEL WEIGHT IN COMMON WHEAT DUE TO BLACK-POINT DISEASE[1]

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INTRODUCTION

This paper deals mainly with the relative weights of kernels of wheat (*Triticum vulgare* Vill.) showing the presence of "black point" and those free from this infection. The main study was made upon kernels from individual wheat plants from the F_5 population resulting from the cross Reward × ((Kota × Marquis) × Hope). The wheat, grown at Fargo in 1933, was planted on May 5, which was about 2 weeks later than the earliest date available for planting in the nursery where it was grown.

A sample of the infected kernels was submitted to H. B. Humphrey, of the Bureau of Plant Industry, United States Department of Agriculture. Dr. Humphrey reported that about one half of the kernels were infected with a strain of *Alternaria*, while the remainder were clearly and positively infected with *Helminthosporium sativum* Pam., King, and Bak. Black point was much in evidence in a number of common wheats in 1933 in the nursery, the percentage of infection varying decidedly with the breeding of the wheat and also, evidently, with the time of planting.

In previous reports upon black point, durum wheats have been held to be most commonly infected (Evans [2] and Weniger)[3] although the infection has not been at all rare upon common wheats. Weniger reports that *Helminthosporium* may produce different types of infection. She mentions head blighting characterized by typical glume lesions and empty spikelets, the latter often occurring several in a group, or as individual empty spikelets (or florets) in any portion of the head. In addition, the black-point type of infection is mentioned as occurring on the seeds. Christensen [4] shows that *H. sativum* attacks different parts of the wheat plant, producing various types of lesions.

According to Christensen the various *Helminthosporium* lesions in wheat or other plants result from repeated inoculations, and Stakman holds that the disease is not systemic.[5] The lesions of black point must be induced then by local spore infection in the ovules. Evidently the time of infection, with regard to the stage of the ovule, and the circumstances attending the development of the fungus until the ripening of the seed, have not been closely studied. Henry [6]

[1] Received for publication Feb. 9, 1934; issued July 1934. Paper no. 8 of the Journal series of the North Dakota Agricultural Experiment Station.

[2] EVANS, N. S. "BLACK POINT" OF WHEAT. (Abstract) Phytopathology 12: 34. 1922.

[3] WENIGER, W. DISEASES OF GRAIN AND FORAGE CROPS IN NORTH DAKOTA. N.Dak. Agr. Expt. Sta. Bull. 255, 97 pp., illus. 1932. (Revision of Bull. 166.)

[4] CHRISTENSEN, J. J. STUDIES ON THE PARASITISM OF HELMINTHOSPORIUM SATIVUM. Minn. Agr. Expt. Sta. Tech. Bull. 11, 42 pp., illus. 1922.

[5] STAKMAN, L. J. A HELMINTHOSPORIUM DISEASE OF WHEAT AND RYE. Minn. Agr. Expt. Sta. Bull. 191, 18 pp., illus. 1920.

[6] HENRY, A. W. ROOT-ROTS OF WHEAT. Minn. Agr. Expt. Sta. Tech. Bull. 22, 71 pp., illus. 1924.

has shown that high temperatures are favorable for the development of *Helminthosporium*, and moisture conditions are of course important.

EXPERIMENTAL WORK

While taking notes on samples of grain threshed from individual plants, the writer was struck by the apparent differences in the size of kernels from any one plant, depending on whether the kernels showed the presence of black point or whether they were free from the infection. Certain of the plants had the weights in grams per 1,000 kernels shown in table 1.

TABLE 1.—*Weight of healthy wheat kernels and of kernels diseased with black point*

Plant no.	Weight per thousand kernels—	
	Healthy	Black-point
	Grams	*Grams*
48.12.247	27.1	33.1
48.12.257	30.7	35.9
48.12.259	26.2	33.9
48.12.288	30.2	36.1
48.12.298	32.2	38.4

The infected kernels in these instances weighed about 20 percent more than the kernels showing no infection.

Of the plants in the group studied in detail, about one half were eliminated in the field because of sterile florets. The remaining plants showed a small amount of floret sterility, but with the exception of 1 or 2 spikes, perhaps no more than might be expected in view of the extreme heat which had prevailed.

Sixteen plants were used in the study of relative weights of kernels. The kernels were carefully removed from the spike and laid in order in depressions cut in paraffin blocks and then weighed to fifths of milligrams. The 16 plants had 63 spikes and 2,030 kernels.

A cursory examination suggested that the differences in kernel weight might be ascribed to a differential incidence of black point on the different kernels of the spikelet relative to position. While the third kernel of the spikelet, when present, showed lesions less commonly than the basal kernels, it became evident that this did not entirely explain the differences.

TABLE 2.—*Number and weight of black-point and healthy kernels found paired in the spikelet, unpaired basal kernels, and kernels of the third floret*

Item	Black-point kernels		Healthy kernels		Excess weight of diseased over healthy kernels
	Number	Average weight	Number	Average weight	
		Milligrams		*Milligrams*	*Milligrams*
Paired	344	39.0±0.17	1,036	36.3±0.13	2.7±0.21
Unpaired	34	35.0± .71	191	32.5± .37	2.5± .80
Third floret	19	31.0± .63	406	27.1± .20	3.9± .66
Total or average	397	38.3± .18	1,633	33.5± .12	[a] 4.7± .22

[a] This value of 4.7 is simply the difference of the weighted means in columns 3 and 5.

The kernels, both those infected with black point and the healthy ones, were placed in three categories. Kernels found paired in the two basal florets comprised the major group; kernels of the third floret, the second group; and unpaired kernels of the basal florets, the third group. The number of kernels, and the average weight per kernel in each of the three classes, and the total weight, are given in table 2.

The elimination of the kernels found in the unpaired and in the third florets reduces the average difference in weight per kernel from 4.7 to 2.7 mg, but even this difference is highly significant as the odds are 3.3×10^{17} to 1 against the probability that a deviation as large as, or larger than, the one indicated could have arisen from random sampling. In the comparison shown in table 2, of the kernels of the third floret, the kernels of the basal spikelet which were significantly below the average in weight and were not infected, are included. Omitting the kernels of the basal spikelet, the difference in weight is still 3.7 ± 0.66 mg. The difference in weight in the unpaired basal kernels is 2.5 ± 0.8 mg and is significant. Considering the 16 plants individually, the black-point kernels of 15 of the plants were the heavier, and the difference was significant in 9 of the 15 cases. In the exceptional case the healthy kernels had the greater weight, but the difference was not significant.

STUDY OF KERNEL WEIGHT ALONG THE SPIKE

In studying the paired kernels attention was given to the disposition of the black-point and healthy kernels along the spike. The results for the 16 plants are summarized in table 3.

TABLE 3.—*Distribution along the spike of black-point and healthy kernels, in pairs, the differences in kernel weight and percentage distribution of kernel weight*

Spikelet no.	Black-point				Healthy				Differences in weight between diseased and healthy kernels		Healthy kernels
	Kernels		Kernel weight per spikelet	Distribution of kernel weight	Kernels		Kernel weight per spikelet	Distribution of kernel weight			
	Number	*Percent*	*Milligrams*	*Percent*	*Number*	*Percent*	*Milligrams*	*Percent*	*Milligrams*	*Percent*	*Percent*
1	27	7.9	35.7±0.55	7.2	145	14.0	32.9±0.30	12.7	2.9± .61	−5.5	84.4
2	52	15.1	40.1± .33	15.5	156	15.1	37.3± .26	15.5	2.8± .42	.0	75.0
3	61	17.7	40.6± .33	18.5	157	15.1	39.2± .26	16.4	1.4± .42	2.1	72.1
4	75	21.8	41.0± .26	23.0	151	14.6	38.8± .27	15.6	2.2± .38	7.4	66.9
5	68	19.8	39.3± .33	19.9	148	14.3	38.0± .33	15.0	1.4± .47	4.9	68.5
6	39	11.3	35.4± .61	10.3	155	15.0	34.9± .34	14.4	.5± .70	−4.1	79.9
7	16	4.7	35.2±1.26	4.2	88	8.5	32.3± .44	7.6	2.9±1.34	−3.4	84.6
8	6	1.7	31.7±1.48	1.4	32	3.1	29.1± .77	2.5	2.6±1.67	−1.1	84.2
9	0	.0	.0	.0	4	.4	34.2	.4	....	−.4	100.0
Total or average	344	100.0	39.0± .17	100.0	1,036	100.1	36.2± .13	100.1	2.8± .21	−.1	.....

From spikelet 1 (basal) to 6 the percentage distribution of the healthy kernels approaches uniformity, varying but 1.1 percent, while the distribution of black-point kernels over the same spikelets shows a range of 13.9 percent. The higher percentages for black-point kernels are found in the center of the spike, and it is there that the heavier healthy as well as black-point kernels are found. The aver-

age weights per kernel for each spikelet are multiplied by the corresponding percentage distributions, and these results, for the two groups, reduced to percentages, are found in columns 5 and 9. When the paired kernels in the two basal florets are studied, it is found that the heavier kernels are those which carry the greater amount of black point. The net results of this difference in weight are indicated in column 10.

The middle kernels of the spike are fertilized first, but the total time for blooming was quite certainly not over 3 days, as the weather was hot and dry. This earlier fertilization may have been responsible in part for a better nutrition of the central kernels and their consequent greater weight. The fungus may have found easier access to the ovules receiving the better nutrition. The time element may have been a factor influencing the place of infection on the spike, but it is doubtful whether the entire difference in weight can be attributed to this difference in time of infection.

It is seen in table 3 that differences in weight between the black-point and healthy kernels are found regularly in each spikelet. When the probable errors were calculated the differences were found to be statistically significant in the five lower spikelets. The few black-point kernels in 2 of the 3 upper spikelets resulted in probable errors too large for the differences to be significant although the absolute differences are relatively large. The sixth spikelet showed a small difference in weight in favor of the black-point kernels. The differences in the standard deviations are very generally contrary to those of the means, in that the distributions of the healthy kernels show greater variability than do those of the black-point kernels. The coefficients of variability of the two series, black point and healthy, for the eight spikelets are shown in table 4.

TABLE 4.—*Coefficients of variability of weights of the basal pair of kernels in the two groups, black point and healthy*

Spikelet no.	Coefficient of variability			Spikelet no.	Coefficient of variability		
	Black-point kernels	Healthy kernels	Difference		Black-point kernels	Healthy kernels	Difference
1	11.70±1.11	16.44±.67	4.74±1.30	5	10.27±.60	15.93±.64	5.66±.88
2	8.66±.58	12.69±.49	4.03±.76	6	15.80±1.25	17.92±.71	2.12±1.44
3	9.32±.57	12.24±.46	2.92±.73	7	20.63±2.56	18.67±.99	−1.96±2.75
4	8.08±.45	12.46±.49	4.38±.67	8	15.42±3.36	21.90±.97	6.48±3.51

In spikelets 1 to 5 the distribution of the healthy kernels has a variability significantly greater than that of the black-point kernels. If the two series of distributions are merged so that the distribution of the total healthy can be compared with the distribution of the total black point it becomes evident where the greater variability of the healthy-kernel series originates. The two distributions expressed in percentages are shown in table 5.

The sums of the positive and negative differences are necessarily equal. The healthy kernels show an excess of variates in the lower weight classes. This is suggested by the two means (table 2). The excess of distribution of the healthy kernels extends over 11 of the lower weight and 1 of the higher weight classes, while the correspond-

ing deficiency is confined to 6 classes in the higher ranges. Skewness is negative in both cases but more pronounced in the case of the healthy kernels. The greater variability shown in table 5 in the healthy kernels characterizes most of the spikelets and especially those which have the greater numbers of kernels.

TABLE 5.—*Percentages of healthy and black-point kernels in the various weight classes*

Item	Percentage of kernels in weight class indicated									
	16 to 17 mg	18 to 19 mg	20 to 21 mg	22 to 23 mg	24 to 25 mg	26 to 27 mg	28 to 29 mg	30 to 31 mg	32 to 33 mg	34 to 35 mg
Healthy kernels	0.4	0.6	1.2	1.5	2.4	4.0	4.1	6.7	8.0	11.1
Black-point kernels	.3	.3	.0	.0	.6	.6	1.7	4.6	4.1	7.6
Difference	.1	.3	1.2	1.5	1.8	3.4	2.4	2.1	3.9	3.5

Item	Percentage of kernels in weight class indicated								
	36 to 37 mg	38 to 39 mg	40 to 41 mg	42 to 43 mg	44 to 45 mg	46 to 47 mg	48 to 49 mg	50 to 51 mg	Total
Healthy kernels	12.3	13.7	15.5	9.0	6.2	2.4	0.8	0.2	100.1
Black-point kernels	11.6	18.0	20.1	15.1	10.2	4.1	.6	.6	100.1
Difference	.7	−4.3	−4.6	−6.1	−4.0	−1.7	.2	−.4	.0

A study was made of the kernel weight of the first and second kernels per spikelet of spikes with nearly or quite healthy kernels. It was not evident that the lower kernel differed in weight from the kernel standing second in position. It follows, then, that the differentiation in weight of kernel with respect to incidence of black point upon the two lower kernels per spikelet is not due to the position of the kernel in the spikelet, associated with normal weight differences.

In any plant, on an average, the black-point kernels are heavier than those evidently free from disease. In part this weight difference may be ascribed to kernel position. Certain kernels of the spike are so located that their weight is decidedly less than that of kernels differently located. There is a differential in incidence of disease with regard to these two kernel groups. Thus weight differences of healthy and diseased kernels may be ascribed to weight differential due to locality in the spike, combined with a differential of disease incidence. But beyond this, between healthy and diseased kernels, weight differences are found which are not due to location in the spike, as is shown in table 2. Finally, a comparison, or series of comparisons, is to be found in the paired kernels of the basal florets of each spikelet. In each of the eight spikelets, the black-point kernels are the heavier (table 3). In these instances, also, the heavier weight of the black-point kernels is not due to any deviation from the normal in kernel size in conjunction with differences in incidence of disease.

The question arises whether the normally larger kernels of the spike were attacked by *Helminthosporium* (and *Alternaria*) or whether, when the young kernels of a group of potentially the same mature size were attacked, such kernels were somehow stimulated to develop a larger

amount of endosperm[7] than if no infection had taken place. It appears that both factors are responsible for the greater weight of the black-point kernels in the spike and in the plant.

CORRELATION RESULTS

It was possible to calculate correlation coefficients between percentages of black point and yield in the 1933 crop. In one instance a considerable number of yields were calculated from 5-foot rows. These rows were planted with F_5 selections from the cross Ceres × Hope-Florence; a single mother plant seeded one row. The stands of grain varied more or less; before harvest, notes were taken on the stand, and the yields were corrected to a uniform stand. The errors involved in this correction were thought to be less than those which would arise from uncorrected yields. Countings were made on the threshed samples of the frequency of the occurrence of black point and the percentages estimated. The weight per 1,000 kernels was also determined. The means and the standard deviations of these three characters calculated from 267 variates are shown in table 6.

TABLE 6.—*Correlations[a] between yield of wheat, weight of black-point kernels, and percentage of black-point kernels in the 1933 crop*

Item	Yield per acre	Weight of 1,000 kernels	Black-point kernels
	Bushels	*Grams*	*Percent*
Mean	34.45±0.31	37.14±0.09	21.98±0.48
Standard deviation	7.57± .22	2.06± .06	11.63± .34

[a] The 3 correlation coefficients calculated were: Black point and yield, 0.22±0.04; yield and 1,000-kernel weight, 0.32±0.04; black point and 1,000-kernel weight, 0.17±0.04.

None of these coefficients is very high, but the first one, which perhaps is of greatest interest, is about five times the probable error. Evidently there is a positive relationship between the presence of the infection and the yield, as secured. When the weight per 1,000 kernels is held constant the partial correlation between yield and black point is 0.18 ± 0.04. This still shows some significance.

In the cross first mentioned in this paper it was possible to calculate correlations similar to those given, except that weight of grain per plant was used instead of yield. The coefficients are as follows:

Black point and grain yield per plant	−0.09±0.04
Yield per plant and 1,000-kernel weight	.27± .04
Black point and 1,000-kernel weight	.32± .03

When the weight per 1,000 kernels is held constant the correlation between black point and grain yield per plant becomes essentially zero. The total correlation between these characters is negative but not significant. Correlation of fairly high significance is shown between black point and 1,000-kernel weight. This is in keeping with the results shown in the earlier part of this paper.

[7] As the endosperm in a normal kernel of wheat comprises about 85 percent of the total weight it is fair to presume that the excess weight of the diseased kernels would be distributed mainly to the endosperm. The black-point kernels appeared to be normal in shape. Any contribution to the greater weight of the black-point kernels made by the substance of the invading organism must have been negligible.

DISCUSSION

More exact studies as to the causal relationships with regard to the various external factors are greatly desired. It is known that the epidemic of black point in 1933 was associated with high temperatures and low seasonal moisture conditions. The wheats studied, started heading about June 22, and blossoming was probably well under way by June 25. A comparison of temperatures and rainfall for the last 5 days of June and the first 15 days of July are shown in table 7.

TABLE 7.—*Temperatures and precipitation for the last 5 days of June and the first 15 days of July 1933*[a]

Item	June 26–30	July 1–5	July 6–10	July 11–15	Total
Temperature:	° *F.*	° *F.*	° *F.*	° *F.*	
Average daily mean	75	75	73	74	
Normal	67	68	67	67	
Excess in 1933	8	7	6	7	
Precipitation:	*Inches*	*Inches*	*Inches*	*Inches*	*Inches*
Total in 1933	1.65	.0	.81	.17	2.63
Normal	.86	.77	.47	.59	2.69

[a] Data are from the official records of the United States Weather Bureau. The normal values of precipitation were taken from the Monthly Weather Review, Vol. 58, Suppl. No. 34, p. 59, May 1930.

The daily temperatures were greatly in excess of the normal for the whole 20 days and particularly during the last 5 days of June. The season was unusually dry, but from June 26 to 30, 1.65 inches was recorded, which was decidedly in excess of the expected amount for the 5-day period. Thus for a short period, in the early life of the wheat seed, conditions of high temperature and high rainfall obtained. It is quite unknown, of course, whether the weather conditions varied in such a manner during the period of infection as to account for the differences in incidence of black point which were shown to exist between basal kernels and kernels of the third floret and also between the mid-spike kernels and those near the base and near the tip of the spike. It is not conceivable that any differentiation in weight due to position could be brought about within a single spikelet or in the third-floret kernels within the spike, as was the case in several instances.

On the other hand there seems to be no report in the literature that a seed infected by a fungus is stimulated, and consequently increases in growth. If stimulation resulted in this case, an increased amount of endosperm must have been laid down, but there seems to be little or no information as to any causal relationship which might have brought this about.

SUMMARY

A heavy infection of black point, caused largely by *Helminthosporium sativum* Pam., King, and Bak., was found on various common wheats at Fargo in 1933. On any one plant, in the hybrids studied, the black-point kernels generally were definitely heavier than the kernels showing no evidences of infection.

This difference in weight can be ascribed in part to a difference in infection of kernels differing normally in size because of position in the spike. Third-floret kernels and end-spike kernels carried less infection than the heavier mid-spike kernels.

Within any kernel group of the spike, such as the third-floret group, the black-point kernels were significantly heavier than the noninfected kernels. The obvious conclusion seems to be that a portion of the weight differences results from a stimulation of the development of the endosperm following the entrance of the fungus into the developing ovule.

In one experiment, a coefficient of correlation of 0.22 ± 0.04 was found between the percentage of black point and the yield in 5-foot rows and a coefficient of 0.32 ± 0.04 was found between black point and weight of 1,000 kernels. In a study of individual plants resulting from a different cross, a correlation of -0.09 ± 0.04 was secured between the percentage of black point and the weight of the grain per plant and a correlation of 0.32 ± 0.03 was obtained between percentage of black point and the weight per 1,000 kernels.

A STATISTICAL STUDY OF THE RELATIONSHIPS BETWEEN THE CONSTITUENTS OF MILK [1]

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INTRODUCTION

The quantitative relationships of the fat, protein, total solids, and solids not fat in cow's milk have been extensively investigated by Overman, Sanmann, and Wright (*13*),[2] Gaines (*6*), Cranfield, Griffiths, and Ling (*4*, *5*), and others. The purpose of this study is to contribute further to the subject in general, but especially to extend the consideration into the field of the inorganic constituents, which have received comparatively little attention.

Many studies of the relations existing between the different constituents of milk have been undertaken from the commercial point of view, especially with reference to the detection of adulteration, the establishment of legal standards, and the enrichment of milk, particularly in fat; and other studies have been made on the origin of the various constituents of milk, the methods of their secretion, the factors affecting their elaboration, and the interrelationship of these constituents. In the present study, which is statistical in method, the immediate objective has been to discover relationships of constituents which might subsequently be subjected to intensive investigation from the physiological point of view.

The data on which this paper is based were obtained from analyses of the milk of 12 Holstein-Friesian cows during an entire period of lactation. The animals were maintained under uniform conditions of care and feeding, without pasture, and were kept indoors, except for short daily periods of controlled exercise.

In relation to feeding treatment the cows were divided into two groups, one of which received timothy hay, corn silage, and concentrate mixture; the other, alfalfa hay, corn silage, and concentrate mixture. The protein content of the rations of the two groups was approximately equalized by varying the composition of the concentrates. Within both of the groups 2 cows were given a supplement of pulverized limestone, 2 a supplement of commercial bone meal, while 2 received no mineral supplement.

No evidence was found that the composition of the rations affected the composition of the milk in any characteristic manner. The analyses of the milk, therefore, were combined into a single group for the present study.

The usual seasonal variation in the composition of milk, dependent on changes in the ration, was not brought out by the method employed in this investigation, but the changes in the composition of milk which occur with the progress of lactation were observed, and these contributed to the statistical constants determined.

[1] Received for publication Feb. 21, 1934; issued July 1934. Published as Technical Paper no. 638 of the Institute of Animal Nutrition.

[2] Reference is made by number (italic) to Literature Cited, p. 1032.

The relationships reported are those of fat, energy, protein, total solids, solids not fat, lactose, ash, calcium, phosphorus, magnesium, chlorine, sodium, and potassium.

EXPERIMENTAL METHODS

The data treated represent 134 samples of milk which were, in greater part, composites of aliquots of each milking for 28-day periods. The time of calving, or of the end of the period of lactation, however, made certain of the milk-sampling periods shorter than the usual 28 days.

The method of preserving the milk, and the methods and results of the determinations of energy, fat, protein, total solids, and solids not fat, have been reported in a previous publication by Kahlenberg and Voris (*10*).

Ash, sodium, and potassium were determined by the official methods of the Association of Official Agricultural Chemists (*2*).

Calcium and magnesium were determined by the McCrudden method (*11, 12*), combined with the filtration and titration technic suggested by Halverson and Schulz (*8*).

Phosphorus was determined gravimetrically by the Neumann-Pemberton wet combustion method (*15*).

Chlorine was determined by the official method of the Association of Official Agricultural Chemists (*2*) except that 0.05 normal silver nitrate solution and 0.03 normal potassium thiocyanate were used instead of 0.1 normal silver nitrate and potassium thiocyanate as specified.

The percentage of lactose was obtained by subtracting the sum of the percentages of ash and protein from the percentage of solids not fat.

The statistical methods were, in general, standard procedures, in the form used by Harris and Benedict (*9*), with supplementary use of Pearson's tables (*14*).

PRESENTATION OF DATA AND DISCUSSION OF RESULTS

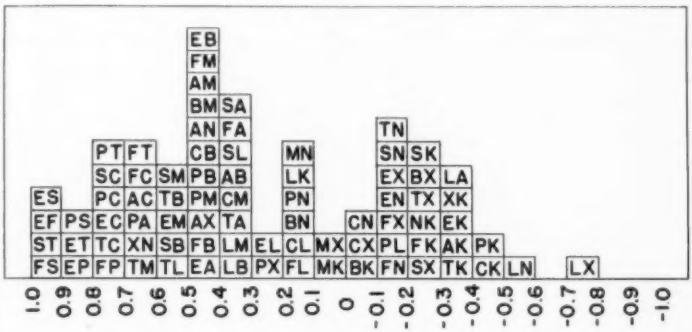
Table 1 summarizes the analytical data arranged in the order of increasing percentage of fat, by giving the mean percentage of each constituent corresponding to intervals of 0.2 percent in the mean fat content. The means, standard deviations, and coefficients of variation, with their respective probable errors, are given in table 2; the coefficients of correlation between the milk constituents are recorded in table 3; and the correlation coefficients are arranged with respect to magnitude and statistical significance, in the form of a frequency distribution, in figure 1.

VARIABILITY

It has been generally conceded in discussions of the composition of milk that fat is one of the most variable, if not the most variable, constituent. However, the individual inorganic constituents have not generally been taken into account—the ash usually being considered as a single ingredient—and the samples of milk have not usually been obtained from animals under conditions of experimental control.

The coefficients of variation in table 2 show that the sodium, the chlorine, and the magnesium content of milk varies much more than

does the fat, and that protein varies as much as does fat. It may be that the fat content of milk is more easily affected by disturbing conditions than is the protein content, and that under the uniform conditions maintained throughout this experiment fat was less variable than it is under ordinary feeding practices.


EB
FM
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PT FT CB SL MN SN SK
SC FC SM PB AB LK EX BX LA
ES PC AC TB PM CM PN EN TX XK
EF PS EC PA EM AX TA BN CN FX NK EK
ST ET TC XN SB FB LM EL CL MX CX PL FK AK PK
FS EP FP TM TL EA LB PX FL MK BK FN SX TK CK LN LX
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0 -0.1 -0.2 -0.3 -0.4 -0.5 -0.6 -0.7 -0.8 -0.9 -1.0

FIGURE 1.—Distribution of correlation coefficients between quantities of milk constituents: *F*, fat; *E*, energy; *P*, protein; *S*, total solids; *T*, solids not fat; *L*, lactose; *A*, ash; *C*, calcium; *B*, phosphorus; *M*, magnesium; *X*, chlorine; *N*, sodium; and *K*, potassium.

TABLE 1.—*Summary of analytical data, giving average percentages of each milk constituent when mean fat content is aranged in 0.2 percent classes*

Fat range (percent)	Samples	Fat	Nitrogen	Protein	Total solids	Solids not fat	Energy per gram	Lactose
	Number	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Calories*	*Percent*
2.60 to 2.79	6	2.70	0.424	2.71	10.85	8.15	586	4.71
2.80 to 2.99	13	2.93	.434	2.77	11.37	8.44	615	4.93
3.00 to 3.19	26	3.10	.447	2.85	11.79	8.69	643	5.12
3.20 to 3.39	34	3.29	.470	3.00	12.08	8.79	666	5.06
3.40 to 3.59	13	3.46	.498	3.25	12.46	9.00	690	5.01
3.60 to 3.79	18	3.72	.510	3.31	12.68	8.96	716	4.93
3.80 to 3.99	10	3.89	.513	3.27	12.98	9.09	732	5.05
4.00 to 4.19	7	4.09	.569	3.63	13.49	9.40	774	5.01
4.20 to 4.39	3	4.25	.571	3.65	13.93	9.68	793	5.24
4.40 to 4.60	4	4.50	.600	3.83	14.40	9.90	825	5.26

Fat range (percent)	Samples	Ash	Sodium	Potassium	Calcium	Magnesium	Phosphorus	Chlorine
	Number	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
2.60 to 2.79	6	0.742	0.081	0.143	0.094	0.013	0.085	0.132
2.80 to 2.99	13	.741	.063	.146	.103	.012	.091	.110
3.00 to 3.19	26	.737	.054	.149	.106	.012	.093	.100
3.20 to 3.39	34	.743	.055	.143	.109	.013	.094	.108
3.40 to 3.59	13	.770	.076	.140	.116	.013	.097	.117
3.60 to 3.79	18	.775	.058	.128	.121	.013	.097	.109
3.80 to 3.99	10	.768	.068	.144	.115	.015	.101	.098
4.00 to 4.19	7	.768	.059	.126	.123	.014	.095	.110
4.20 to 4.39	3	.790	.082	.121	.130	.016	.105	.109
4.40 to 4.60	4	.810	.066	.139	.128	.019	.111	.095

Among the individual inorganic constituents, sodium seems to be the most variable. This may be only apparent, the variability of the data being due in part to difficulties encountered in analysis; but chlorine, with which sodium shows the closest correlation, and which is not particularly difficult to determine, also shows a variability

strikingly greater than that of any of the other constituents except magnesium. Therefore, inasmuch as sodium and chlorine are largely in combination with each other, sodium chloride is presumably the most variable constituent of milk. Possible explanations of this variability are suggested in the discussion of the correlation coefficients.

TABLE 2.—*Means, standard deviations, and coefficients of variation of constituents of milk*

Constituent	Means	Standard deviations	Coefficients of variation
Fat	3.4095±0.0245	0.4207±0.0173	12.34±0.52
Protein	3.0770± .0224	.3838± .0158	12.47± .52
Total solids	12.2713± .0481	.8250± .0340	6.72± .28
Solids not fat	8.8619± .0277	.4752± .0196	5.36± .22
Energy	680.075 ±3.299	56.61 ±2.332	8.32± .35
Lactose	5.0274± .0181	.3108± .0128	6.18± .26
Ash	.7547± .0024	.0420± .0017	5.57± .23
Sodium	.0576± .0011	.0184± .0008	31.94±1.44
Potassium	.1392± .0010	.0172± .0007	12.36± .52
Calcium	.1117± .0007	.0117± .0005	10.47± .44
Magnesium	.0131± .0002	.0030± .0001	22.90± .99
Phosphorus	.0951± .0005	.0090± .0004	9.46± .39
Chlorine	.1077± .0013	.0216± .0009	20.06± .86

TABLE 3.—*Coefficients of correlation between quantities of milk constituents*

Constituent	Correlation r	Constituent	Correlation r
Fat and—		Total solids and—Continued.	
Energy	0.9502±0.0057	Phosphorus	0.5448±0.0410
Total solids	.9076± .0103	Ash	.3843± .0497
Protein	.7000± .0297	Lactose	.3667± .0504
Solids not fat	.6885± .0306	Sodium	−.1555± .0569
Calcium	.6646± .0325	Chlorine	−.2064± .0558
Magnesium	.4690± .0455	Potassium	−.2932± .0533
Phosphorus	.4166± .0482	Solids not fat and—	
Ash	.3823± .0498	Total solids	.9382± .0070
Lactose	.1264± .0573	Energy	.8565± .0155
Sodium	−.1027± .0576	Protein	.7868± .0222
Chlorine	−.1194± .0574	Calcium	.7670± .0240
Potassium	−.2242± .0553	Fat	.6885± .0306
Energy and—		Magnesium	.6083± .0367
Total solids	.9817± .0021	Phosphorus	.5813± .0386
Fat	.9502± .0057	Lactose	.5320± .0418
Solids not fat	.8565± .0155	Ash	.3362± .0517
Protein	.8101± .0200	Sodium	−.1777± .0564
Calcium	.7698± .0237	Chlorine	−.2506± .0546
Magnesium	.5612± .0399	Potassium	−.3061± .0528
Phosphorus	.4787± .0449	Lactose and—	
Ash	.4125± .0484	Solids not fat	.5320± .0418
Lactose	.2491± .0547	Total solids	.3667± .0504
Sodium	−.1201± .0574	Magnesium	.3281± .0520
Chlorine	−.1452± .0570	Phosphorus	.3005± .0530
Potassium	−.3252± .0521	Energy	.2491± .0547
Protein and—		Potassium	.1767= .0565
Total solids	.8760± .0136	Calcium	.1306± .0573
Energy	.8101± .0200	Fat	.1264± .0573
Solids not fat	.7868± .0222	Protein	−.1134± .0575
Calcium	.7728± .0235	Ash	−.3891± .0494
Fat	.7000± .0297	Sodium	−.5007± .0437
Ash	.6194± .0359	Chlorine	−.7030± .0295
Phosphorus	.4354± .0472	Ash and—	
Magnesium	.4262± .0477	Calcium	.06378± .0346
Chlorine	.2197± .0555	Protein	.6194± .0359
Sodium	.1445± .0571	Magnesium	.4579± .0460
Lactose	−.1134± .0575	Sodium	.4511± .0464
Potassium	−.4934± .0441	Chlorine	.4185± .0481
Total solids and—		Energy	.4125± .0484
Energy	.9817± .0021	Total solids	.3843± .0497
Solids not fat	.9382± .0070	Fat	.3823± .0498
Fat	.9076± .0103	Phosphorus	.3564± .0509
Protein	.8760± .0136	Solids not fat	.3362± .0517
Calcium	.7793± .0229	Potassium	−.3219± .0522
Magnesium	.5893± .0380	Lactose	−.3891± .0494

TABLE 3.—*Coefficients of correlation between quantities of milk constituents*—Con.

Constituent	Correlation r	Constituent	Correlation r
Calcium and—		Chlorine and—	
Total solids	0.7793±0.0229	Sodium	0.6109±0.0365
Protein	.7728± .0235	Ash	.4185± .0481
Energy	.7698± .0237	Protein	.2197± .0555
Solids not fat	.7670± .0240	Magnesium	.0444± .0582
Fat	.6646± .0325	Calcium	−.0620± .0580
Ash	.6378± .0346	Fat	−.1194± .0574
Phosphorus	.4508± .0464	Energy	−.1452± .0570
Magnesium	.3365± .0517	Total solids	−.2064± .0558
Lactose	.1306± .0573	Solids not fat	−.2506± .0546
Chlorine	−.0620± .0580	Phosphorus	−.2632± .0542
Sodium	−.0785± .0579	Potassium	−.3825± .0497
Potassium	−.4399± .0470	Lactose	−.7030± .0295
Phosphorus and—		Sodium and—	
Solids not fat	.5813± .0386	Chlorine	.6109± .0365
Total solids	.5448± .0410	Ash	.4511± .0464
Energy	.4787± .0449	Magnesium	.1926± .0561
Magnesium	.4519± .0464	Protein	.1445± .0571
Calcium	.4508± .0464	Phosphorus	.1383± .0572
Protein	.4354± .0472	Calcium	−.0785± .0579
Fat	.4168± .0482	Fat	−.1027± .0576
Ash	.3564± .0509	Energy	−.1201± .0574
Lactose	.3005± .0530	Total solids	−.1555± .0569
Sodium	.1383± .0572	Solids not fat	−.1777± .0564
Potassium	−.0095± .0583	Potassium	−.2367± .0550
Chlorine	−.2632± .0542	Lactose	−.5007± .0437
Magnesium and—		Potassium and—	
Solids not fat	.6083± .0367	Lactose	.1767± .0565
Total solids	.5893± .0380	Magnesium	.0382± .0582
Energy	.5612± .0399	Phosphorus	−.0095± .0583
Fat	.4690± .0455	Fat	−.2242± .0553
Ash	.4579± .0460	Sodium	−.2367± .0550
Phosphorus	.4519± .0464	Total solids	−.2632± .0533
Protein	.4262± .0477	Solids not fat	−.3061± .0528
Calcium	.3365± .0517	Ash	−.3219± .0522
Lactose	.3281± .0520	Energy	−.3252± .0521
Sodium	.1926± .0561	Chlorine	−.3825± .0497
Chlorine	.0444± .0582	Calcium	−.4399± .0470
Potassium	.0382± .0582	Protein	−.4934± .0441

The exceptional variability of magnesium which was observed has also been noted by Allen (*1*), but no adequate explanation of this fluctuation is apparent.

Solids not fat show the least variability, followed, in the order of increasing variability, by ash, lactose, total solids, energy, phosphorus, calcium, chlorine, magnesium, and sodium.

CORRELATIONS

In the interpretation of the correlation coefficients any correlations greater than 0.4600, that is, correlations of a magnitude greater than 10 times their probable error, have been considered of definite significance. Correlations of from 0.3130 to 0.4600, which are more than 6 and less than 10 times their probable error, have been designated as of doubtful significance, and correlations of less than 6 times their probable error have not been considered significant.

The following general statements of significant relationships were observed:

(1) As the percentage of fat increases, the energy, and the percentages of total solids, protein, solids not fat, calcium, and magnesium increase.

(2) As the energy value increases, the percentages of total solids, solids not fat, protein, calcium, magnesium, and phosphorus increase.

(3) As the percentage of protein increases, the percentages of total solids, solids not fat, calcium, and ash increase, and the percentage of potassium decreases.

(4) As the percentage of total solids increases, the percentages of solids not fat, calcium, magnesium, and phosphorus increase.

(5) As the percentage of solids not fat increases, the percentages of calcium, magnesium, phosphorus, and lactose increase.

(6) As the percentage of lactose increases, the percentages of sodium and chlorine decrease.

(7) As the percentage of ash increases, the percentage of calcium increases.

(8) As the percentage of chlorine increases, the percentage of sodium increases.

Among the correlations of doubtful significance it is of interest to note that as magnesium increases the percentages of ash, phosphorus, protein, calcium, and lactose increase. It has been noted above that magnesium is significantly correlated with solids not fat, total solids, energy, and fat. Thus, in 9 of the 12 comparisons, magnesium shows a significant, or probably significant, positive correlation. Similarly, calcium shows high positive correlations with total solids, protein, energy, solids not fat, fat, and ash, with possibly significant positive correlations with phosphorus and magnesium.

Among the negative correlations of special interest are those of potassium with protein and calcium; and of lactose, which is a relatively constant constituent, with sodium and chlorine, which are extremely variable. The findings with reference to lactose, sodium, and chlorine are in harmony with the conclusion of Porcher (*16*) that milk with high lactose content is usually low in sodium chloride, and that mainly by variation in the sodium chloride in the milk it is maintained isotonic with the blood.

Gowen and Tobey (*7*) state that the osmotic pressure of milk is very largely determined by the lactose content, whereas the osmotic pressure of blood is relatively little determined by its sugar content but mostly by its salt content, referring to six major osmotically active elements, potassium, sodium, calcium, magnesium, phosphorus, and chlorine. In the maintenance of the isotonicity of blood and milk, the milk of a lower lactose content would have to increase its content of salts in a rather pronounced degree to make up for this deficiency of lactose. Gowen and Tobey propose to compute the osmotic pressure from the millimolar concentration of lactose and ash found in the milk. From the present data lactose does reveal a negative correlation with ash, but among the individual elements only sodium and chlorine were found negatively correlated with lactose.

Blackwood and Stirling (*3*) consider that by far the largest part of any osmotic change taking place during milk secretion is to be accounted for by the synthesis of protein and carbohydrate from glucose and amino acids. They consider that during milk secretion a transudate is separated from the blood, having the same osmotic pressure as blood, and that during synthesis water is returned to the blood to maintain the tonicity of the cell fluid.

In the present study protein shows no significant correlations, positive or negative, with lactose and sodium chloride; and these correlations, as contrasted with the significantly negative correlation

of lactose and sodium chloride, suggest a dissimilar relation of protein and lactose in the maintenance of the isotonicity of the blood and milk.

The narrow range of variation in the hydrogen-ion concentration of freshly drawn milk, between 6.50 and 6.65, is probably maintained chiefly through the phosphates and casein present. It was shown by Rice and Markley (*17*) that the hydrogen-ion concentration of milk of high acidity, as determined by titration, is affected by the addition of acid or alkali to a much lesser extent than is the hydrogen-ion concentration of milk with low acidity. Their explanation is that in high-acid milk the phosphate and casein are present in greater quantities than in low-acid milk, and that the buffer action is greater in the former case than in the latter. The negative correlation of potassium and protein is in harmony with this conclusion, and since calcium is largely associated with protein the negative correlation of potassium and calcium seems to be a part of the same picture. However, there is no correlation, either positive or negative, between potassium and phosphorus. In fact, potassium shows no significant positive correlation with any of the constituents determined, though it may be correlated with undetermined constituents, for instance, with citric acid.

Wright (*18*) has shown experimentally that $CaHPO_4$ in solutions of calcium caseinate may be held in stable colloidal solutions up to the concentrations of calcium and phosphorus found in milk. He suggests that the calcium and phosphorus which diffuse from the blood into the mammary gland may thus be held in colloidal combination by the casein in the milk cells. Alkali renders this combination unstable, with which fact the negative correlations of potassium with protein and calcium, as found in this study, seem to be in harmony. This calls attention to the slight degree of correlation found between potassium and phosphorus, and suggests that phosphorus is associated with other constituents than calcium and casein—which is borne out by the fact that the ratio of phosphorus to calcium in calcium caseinate is 0.8, and in $CaHPO_4$ it is 0.775, whereas the ratio between the average percentages of phosphorus to calcium found in milk in the present study is 0.851.

In consideration of the questionable significance of correlations as evidence however, no effort will be made to interpret these observations closely. Correlations can only suggest, and the facts as to origin and relationships of the constituents of milk must be established by evidence of more direct and less equivocal character.

SUMMARY

Statistical data based on the analyses of 134 samples of Holstein-Friesian milk representing one entire lactation period for each of 12 cows are reported. The analyses include fat, energy, protein, total solids, solids not fat, lactose, ash, calcium, phosphorus, magnesium, chlorine, sodium, and potassium. The means, standard deviations, and coefficients of variation of each constituent with their corresponding probable errors, are reported, and the coefficients of correlation of each constituent with every other constituent are given.

LITERATURE CITED

(1) Allen, L. A.
1931. The mineral constituents and citric acid content of milk. Jour. Dairy Research 3: 1–51, illus.

(2) Association of Official Agricultural Chemists.
1925. Official and tentative methods of analysis. Compiled by the committee on editing methods of analysis. Revised to July 1, 1924. Ed. 2, 535 pp., illus. Washington, D.C.

(3) Blackwood, J. H., and Stirling, J. D.
1932. The absorption of milk precursors by the mammary gland V. Physicochemical aspects of milk secretion. Biochem. Jour. 26: [1127]–1137.

(4) Cranfield, H. T., Griffiths, D. G., and Ling, E. R.
1927. The composition of milk. Part I. Variations in the solids not fat, fat and protein content of cow's milk, and their relationship. Jour. Agr. Sci. [England] 17: [62]–71, illus.

(5) ——— Griffiths, D. G., and Ling, E. R.
1927. The composition of milk. Part II. Variations in the percentage of mineral constituents in cow's milk, and their relationship with the solids not fat and protein content. Jour. Agr. Sci. [England] 17: [72]–93, illus.

(6) Gaines, W. L.
1925. Relative rates of secretion of various milk constituents. Jour. Dairy Sci. 8: 486–496, illus.

(7) Gowen, J. W., and Tobey, E. R.
1931. Studies on milk secretion. The influence of inanition. Jour. Gen. Physiol. 15: 45–66.

(8) Halverson, J. O., and Schulz, J. A.
1920. The M'Crudden gravimetric calcium method modified. Jour. Indus. and Engin. Chem. 12: 77–78.

(9) Harris, J. A., and Benedict, F. G.
1919. A biometric study of basal metabolism in man. 266 pp., illus. Philadelphia.

(10) Kahlenberg, O. J., and Voris, L.
1931. The percentage of fat as a basis for estimating the composition of milk. Jour. Agr. Research 43: 749–755.

(11) McCrudden, F. H.
1910. The quantitative separation of calcium and magnesium in the presence of phosphates and small amounts of iron devised especially for the analysis of foods, urine, and feces. Jour. Biol. Chem. 7: 83–100, 201.

(12) ———
1911. The determination of calcium in the presence of magnesium and phosphates: the determination of calcium in urine. Jour. Biol. Chem. 10: 187–199.

(13) Overman, O. R., Sanmann, F. P., and Wright, K. E.
1929. Studies of the composition of milk. Ill. Agr. Expt. Sta. Bull. 325, pp. 51–174, illus.

(14) Pearson, K.
1924. Tables for statisticians and biometricians. Ed. 2. Cambridge.

(15) Pemberton, H., Jr.
1893. The determination of phosphoric acid by the titration of the yellow precipitate with standard alkali. Jour. Amer. Chem. Soc. 15: 382–395.

(16) Porcher, C.
1923. The sodium chloride content of milk. Lait 3: 11–21. [Abstract in Chem. Abs. 17: 1848. 1923.]

(17) Rice, F. E., and Markley, A. L.
1924. The relation of natural acidity in milk to composition and physical properties. Jour. Dairy Sci. 7: 468–483.

(18) Wright, N. C.
1928. The mechancism of secretion of calcium and phosphorus in milk. Jour. Agr. Sci. [England] 18: [478]–485.

INFLUENCE OF CALCIUM PHOSPHORUS INTAKE ON BOVINE BLOOD[1]

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INTRODUCTION

Phosphorus is present in every cell and fluid of the body. It is vitally concerned with carbohydrate, fat, and some phases of protein metabolism. It is essential in muscular contractions and in the functioning of the central nervous system. It enters into the buffering powers of the blood and other tissues. Phosphorus, together with calcium, plays a fundamental role in bone formation. It is, then, not surprising to find profound physiological and anatomical disturbances occurring in animals on a phosphorus-deficient diet. In cattle such a deficiency may manifest itself by hypophosphoraemia, osteophagia, and osteoporosis, and by osteomalacia in the adult (*15*).[2] In the early stages the consumption of food is usually adversely affected (*26*). On phosphorus-deficient rations the milk yield is usually low (*2*) and there is a marked inhibition of oestrum (*4*). Usually before most of the symptoms appear, hypophosphoraemia manifests itself (*3*, *24*).

Aphosphorosis of cattle is known to occur in many regions (*5*, *25*). It is probably even more widely spread than is known at the present time, where the following conditions occur: The forage is produced on phosphorus-deficient soil and consequently is low in phosphorus; the cattle are kept on too restricted rations, the main constituents of which are deficient in phosphorus; the intake of calcium is excessive in relation to the intake of phosphorus; and the phosphorus requirements are extremely high and hence not met by the ordinary rations, as in the case of the lactating cow.

It has been suggested that the estimation of inorganic blood phosphorus gives an early diagnosis of aphosphorosis and helps to differentiate it from picas having a different origin (*14*, *26*). With sufficient information on the optimum inorganic phosphorus of the blood under varying conditions, it appears probable that blood analyses may be used to discover those animals which are on phosphorus-deficient diets, and hence the proper phosphorus supplement may be prescribed. In this manner, at least in a degree, it may be possible to prevent the modern dairy cow from passing through a mild form of osteoporosis at each lactation. This is an extreme instance and at times may not be due to a deficient ration but rather to an overdeveloped udder outrunning the absorptive capacity of the gut for phosphorus (*6*). However, Huffman and his coworkers (*12*) found that even high-yielding cows (2,000 gallons) can be maintained in mineral equilibrium (positive calcium balance) upon ordinary mineral-rich rations. Nevertheless, the margin is often exiguous, and little may be required to change a positive balance to a negative one.

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[2] Reference is made by number (italic) to Literature Cited, p. 1040.

EXPERIMENTAL ANIMALS AND PROCEDURE

Data reported in this paper represent blood analyses obtained from 40 grade yearling beef steers, averaging 585 pounds in weight and of similar breeding and previous treatment. They were carefully sorted into five groups as nearly alike as possible as to weight, quality, and condition. They were fed for a period of 150 days on the following rations:

Lot 1, pressed beet pulp, beet molasses, alfalfa hay, and salt.
Lot 2, pressed beet pulp, beet molasses, alfalfa hay, and cottonseed cake.
Lot 3, pressed beet pulp, beet molasses, alfalfa hay, and steamed bone meal.
Lot 4, pressed beet pulp, beet molasses, alfalfa hay, and mill-run bran.
Lot 5, pressed beet pulp, beet molasses, alfalfa hay, and ground barley.

The average daily ration consumed by each lot, the daily calcium and phosphorus intakes, and the calcium-phosphorus ratio in the rations are shown in table 1.

Lot 1, receiving beet pulp, molasses, alfalfa, and salt, obtained less than 50 percent of the phosphorus received by those on the ration supplemented with cottonseed cake. The phosphorus intake of lot 5, where barley was given, was also low; that of the remaining lots was high. There were also considerably greater quantities of calcium in the rations of the animals receiving phosphorus supplements. This is especially noticeable in the lot receiving cottonseed cake. The calcium oxide intake in the ration of lot 1 equaled 0.74 percent; of lot 2, 0.69 percent; of lot 3, 0.48 percent; of lot 4, 0.52 percent; and of lot 5, 0.73 percent. All these animals received rations containing more than 0.45 percent of calcium oxide placed as the minimum by the Wisconsin workers (*10*) for dry dairy cows. Phosphorus was low in the rations of lots 1 and 5 when compared with the quantities recommended by Kellner (*13*) and Armsby (*1*).

The animals receiving no phosphorus supplements were getting considerably less than the 30 g of phosphorus pentoxide per day, which Theiler, Green, and Du Toit (*27*) laid down as the minimum for heifers. Where barley was the supplement, the animals were receiving 30 g; all others were receiving considerably more than the minimum requirements.

Theiler, Green, and Du Toit (*26*) conclude that inasmuch as minimum requirements of growth are higher in the case of phosphorus than in the case of calcium, a ratio of calcium oxide to phosphorus pentoxide as high as 3 to 1 is not necessarily disadvantageous. Lots 2 and 3 were receiving rations in which the ratio of calcium oxide to phosphorus pentoxide was 3 to 1. Lot 1 received calcium oxide to phosphorus pentoxide in a ratio of 4.6 to 1 and lot 5 in the ratio of 4.1 to 1. It is evident, therefore, that in lots 1 and 5 phosphorus could have been profitably added. One is prone to ask: Could greater quantities of phosphorus have been added advantageously to the rations of lots 2, 3, and 4? Theiler, Green, and Du Toit (*26*) state that when calcium is low it is probable that a relatively high proportion of phosphorus may facilitate the absorption of calcium and thus reduce the risk of calcium starvation. When phosphorus is low, a relatively high calcium intake may reduce the absorption of phosphorus and in this way increase the danger of aphosphorosis.

TABLE 1.—*Average daily ration consumed, average daily intake of calcium and phosphorus, and the calcium-phosphorus ratio in the rations*

Lot no.	1		2		3		4		5	
Average ration consumed (pounds)	Beet pulp	21.4	Cottonseed cake	1.8	Bone meal	0.1	Mill-run bran	2.7	Ground barley	3.6
	Molasses	2.5	Beet pulp	34.6	Beet pulp	48.6	Beet pulp	42.0	Beet pulp	24.7
	Alfalfa	7.1	Molasses	2.5	Molasses	2.5	Molasses	2.5	Molasses	2.5
	Salt	.06	Alfalfa	6.4	Alfalfa	6.3	Alfalfa	6.3	Alfalfa	6.7
			Salt	.06	Salt	.04	Salt	.04	Salt	.04
						41		.48		.30
Phosphorus as P_2O_5 in ration (grams)		23		48		127		127		125
Calcium as CaO in ration (do)		105		143						
CaO / P_2O_5 (ratio)		4.6		3.0		3.1		2.6		4.1

Samples of blood were collected in the morning before feeding to obviate the action of recently ingested carbohydrates on blood inorganic phosphorus. It was found in preliminary tests in 1932, when this precaution was not taken, that the blood inorganic phosphorus often fell to an extremely low level, probably due to the large quantities of soluble carbohydrates consumed.

The blood was collected from the jugular vein in two 4-ounce bottles. The sample for phosphorus determination was quickly mixed with sodium oxalate, immediately taken to the laboratory, and the plasma centrifuged out. The phosphorus was determined in the plasma by the Youngberg method (*11*); the calcium in the serum of the second sample by the Clark-Collip modification of the Krammer-Tisdal method (*11*). Results are reported as milligrams of phosphorus in 100 cc of blood plasma and milligrams calcium in 100 cc of blood serum (table 2). Samples were taken at the beginning of the experiment and during each succeeding month. This work represents analyses of individual samples and not the composite of three samples taken on successive days as reported by some workers. In this work, however, samples were taken from each of eight animals receiving the same treatment. This should eliminate, to a degree, individual and daily fluctuations in the calcium and phosphorus content of the blood.

EXPERIMENTAL DATA

The average calcium content of the blood of the various lots on January 21, 1933, varied from 12.25 mg per 100 cc of blood serum to 13.16 mg. With few exceptions, there is only a small variation within the different groups (table 2).

TABLE 2.—*Milligrams of calcium per 100 cc of blood serum and of inorganic phosphorus per 100 cc of blood plasma in blood of steers fed different supplements to the ration, Jan. 21 and Feb. 28, 1933*

DATA OF JAN. 21, 1933

Steer no.	Lot 1, no supplement		Lot 2, cottonseed cake			Lot 3, steamed bone meal			Lot 4, mill-run bran			Lot 5, ground barley		
	Ca	P	Steer no.	Ca	P	Steer no.	Ca	P	Steer no.	Ca	P	Steer no.	Ca	P
3	13.50	2.87	1	12.00	2.30	2	12.00	1.89	7	13.50	2.56	5	13.25	2.52
12	12.00	2.71	6	13.00	2.18	4	11.25	2.68	13	12.00	3.16	8	11.75	3.76
22	14.00	3.07	10	11.75	2.47	16	13.50	3.65	21	12.50	2.72	23	12.00	2.78
29	13.75	2.26	15	13.75	2.15	18	13.50	3.40	24	14.00	1.74	28	13.25	2.91
40	13.75	2.54	25	14.00	1.67	32	11.00	3.51	26	11.25	2.93	31	12.50	3.46
41	12.00	2.59	30	13.00	3.10	33	11.25	2.75	34	11.75	2.38	36	10.50	2.96
46	11.50	2.79	42	13.75	2.21	14	12.00	2.47	35	14.00	1.69	44	13.00	3.21
49	13.75	2.24	43	13.75	3.23	48	16.00	2.72	45	13.75	3.73	50	11.75	2.49
Average	13.04	2.63		13.13	2.41		12.56	2.88		12.84	2.61		12.25	3.01

DATA OF FEB. 28, 1933

3	13.3	3.13	1	13.5	4.49	14	13.3	4.74	21	14.0	6.67	50	15.8	3.38
12	13.0	3.25	25	14.3	4.07	48	13.5	6.25	45	14.0	4.55	44	14.0	4.35
22	13.0	4.13	6	13.8	3.54	2	12.8	6.29	34	14.8	4.52	5	15.3	3.02
29	15.0	3.13	43	13.5	4.55	16	12.8	5.71	13	14.0	7.04	23	14.5	3.37
40	13.5	3.73	15	14.0	4.07	18	14.5	4.17	24	14.5	4.39	8	14.8	4.42
41	12.8	3.70	42	14.3	5.38	33	14.3	5.38	26	11.3	5.95	36	15.3	3.31
46	14.0	3.91	10	13.0	4.39	32	13.0	6.58	35	14.5	3.57	28	14.0	3.16
49	13.0	3.85	30	14.0	4.52	4	13.5	5.65	7	14.0	6.62	31	15.3	4.35
Average	13.4	3.60		13.8	4.38		13.5	5.60		13.9	5.41		14.9	3.67

These animals had grazed on the range plants which were grown on the highly calcareous soils of Idaho and consequently were high in calcium. This may account for the high calcium and comparatively low phosphorus content of the animal's blood.

The inorganic phosphorus of the blood varied from 2.41 to 3.01 mg per 100 cc of blood plasma. These results are low when compared with those obtained by Eckles and his coworkers (*5*) on animals receiving sufficient phosphorus and point strongly to the conclusions that all these animals, although on a ration high in alfalfa hay grown on phosphorus-rich soils, would have benefited if given a phosphorus supplement.

The phosphorus-calcium ratio in the blood varied from 1 to 4 in barley-fed steers and from 1 to 5.5 in animals receiving cottonseed cake.

On February 28, 1933, the blood was again analyzed (table 2). A slight increase in the blood calcium had occurred. Even where the animals received the calcium supplements there was an increase of only 1 mg per 100 cc of blood serum, which is in keeping with the findings of Theiler, Green, and Du Toit (*26*). The phosphorus, however, had markedly increased. The animals receiving bone meal and mill-run bran yielded blood phosphorus well above 5 mg per 100 cc of blood plasma. In those animals receiving no phosphorus supplement and those receiving ground barley, the blood phosphorus was lower, being 3.60 and 3.67 mg per 100 cc of blood plasma, respectively. The phosphorus-calcium ratio, while still varying widely in the blood of different lots, was much narrower.

Blood samples were also taken on April 1, April 27, and June 1, 1933. These showed the same regularity in each group, as did the analyses made on January 21 and February 28. Hence, only the averages of these determinations are given (table 3). Each reported result represents the averages of the analyses made on eight different animals receiving the same ration which should rule out individual differences that occur when averages are made from individual animals.

TABLE 3.—*Average milligrams of calcium in 100 cc of blood serum and of inorganic phosphorus per 100 cc of blood plasma in the blood of steers fed different supplements to the ration on given dates*

[Averages represent 8 determinations made on different animals receiving the same rations]

CALCIUM

Date of sampling	Lot 1, no supplement	Lot 2, cottonseed cake	Lot 3, steamed bone meal	Lot 4, mill-run bran	Lot 5, ground barley
1933					
Jan. 21	13.16	13.13	12.58	12.84	12.25
Feb. 28	13.40	13.18	13.50	13.90	14.90
Apr. 1	15.22	12.03	12.31	12.63	12.56
Apr. 27	13.41	12.19	12.19	11.88	12.59
June 1	14.10	13.00	13.00	12.20	12.80

INORGANIC PHOSPHORUS

1933					
Jan. 21	2.63	2.41	2.88	2.61	3.01
Feb. 28	3.60	4.38	5.60	5.41	3.67
Apr. 1	2.67	4.12	5.22	4.52	3.58
Apr. 27	2.60	4.28	4.71	4.52	3.35
June 1	3.11	4.45	4.35	4.98	3.73

When placed on the experimental ration, these cattle were taken from feeds high in calcium. Moreover, all experimental rations carried calcium well above the minimum requirements for such animals; consequently, their blood calcium showed no appreciable variation that could be ascribed to these rations. The greatest variation is shown in the lot receiving no phosphorus supplement. Due to the drop in phosphorus, nature may have attempted to maintain an approximate uniform calcium times phosphorus concentration. Palmer and Eckles (*20*) produced hypercalcemia and hypophosphoraemia in cows by feeding diets low in phosphorus. Had the animals used in the experiments here reported been younger, it is highly probable that greater variations would have occurred in the serum calcium, for it is not easy to raise the concentration of serum calcium far above normal by feeding calcium salts or high calcium-containing feed; it is even more difficult to lower the serum calcium of normal full-grown animals by altering the intake of calcium (*23, v. 1, p. 814*).

The inorganic phosphorus of the blood plasma on January 21, when the cattle were placed on the various rations, was low. There was an increase on February 28, greatest in the case of steers receiving bone meal and least where no phosphorus supplement was given. The concentrations remained quite uniform throughout the remainder of the experiment. Only occasionally did the inorganic phosphorus content reach 5-mg concentration, making it highly probable that all of these animals would have benefitted by an increase in the phosphorus supplement due to the high calcium intake.

DISCUSSION

It has been stated (*23*) that calcium and phosphorus in the circulating plasma, other factors remaining constant, bear a reciprocal relationship to one another. Harnes (*8, 9*) tested this biometrically on the blood of rabbits and found a small negative correlation between the calcium and inorganic phosphorus. Palmer and his coworkers (21) found no correlation between calcium and inorganic phosphorus and consider Harnes' coefficient so small in relation to the probable error as to be mathematically insignificant. Calculations have been made of the correlation coefficients between calcium and phosphorus in the 199 analyses, as reported herein. The formulas given by Pearl (*22*) were used: $r_{12}=\frac{(x_1x_2)}{N\sigma_1\sigma_2}$ and $P\cdot E_{\cdot r}=0.67449\,\frac{1-r^2}{\sqrt{N}}$.

The values for r_{12} on the following dates are:

Jan. 21, -0.1660 ± 0.1037 ($n=40$)
Feb. 28, -0.3333 ± 0.0947 ($n=40$)
Apr. 1, -0.5116 ± 0.0784 ($n=40$)
Apr. 27, -0.5888 ± 0.0696 ($n=40$)
June 1, -0.6096 ± 0.0678 ($n=39$)
On all samples, -0.2264 ± 0.0452 ($n=199$)

Hence, there is a small negative correlation between the inorganic phosphorus and calcium in the blood of steers fed on the rations considered in this work. It becomes greater as the experiment progresses; however, more work is necessary before it can be said to have a biological significance.

There is a close relationship between the phosphorus intake of these animals and the inorganic phosphorus of the blood plasma, which bears out the findings of others (*11*, *19*). Analyses of blood for its inorganic phosphorus content can be used as a measure to determine the phosphorus needs of animals. This, however, requires further work to standardize the factor for optimum concentration under varying conditions. The age of the animal is known to influence the inorganic phosphorus of the blood (*7*), and the conclusion is quite well-founded that gestation is without effect (*16*, *18*). However, the effect of sex and pregnancy upon the normal inorganic phosphorus of the blood is not so well known.

At the end of the 150-day period, the ration of the cattle receiving pressed beet pulp, beet molasses, alfalfa hay, and salt (lot 1) was supplemented with 0.1 pound of steamed bone meal daily. After being on this ration for 30 days, blood samples were taken and analyzed for calcium and inorganic phosphorus. These results are given in table 4.

TABLE 4.—*Milligrams of calcium in 100 cc of blood serum and of inorganic phosphorus in 100 cc of blood plasma in the blood of steers, July 1, 1933.*

Steer no.	Calcium	Phosphorus	Steer no.	Calcium	Phosphorus	Steer no.	Calcium	Phosphorus
40	13.3	3.4	3	14.5	2.4	50	13.0	2.4
6	12.5	3.9	49	13.3	2.0	22	12.5	3.5
34	12.5	3.3	46	12.0	3.9	5	13.0	3.3
12	13.0	3.4						

The calcium of the blood remained quite constant. The inorganic phosphorus of the blood increased; at the close of the experiment, however, it was still far below that of the animals which had received steamed bone meal from the beginning of the experiment. Undoubtedly, due to the deficient ration, other tissues of the body, in addition to the blood, had been depleted of their phosphorus; consequently, time apparently is required to bring the tissues back to normal.

SUMMARY

Forty head of grade beef steers, averaging 585 pounds in weight and of similar breeding and previous treatment, were sorted into 5 groups as nearly alike as possible as to weight, quality, and condition and were fed for 150 days on rations of pressed beet pulp, beet molasses, alfalfa hay, and salt, to which was added for four of the groups a supplement of cottonseed cake, steamed bone meal, mill-run bran, and ground barley, respectively.

Blood samples were taken monthly and analyzed for calcium and phosphorus. Before going on these rations the averages per 100 cc of blood serum and plasma were: Calcium from 12.25 to 13.13 mg and inorganic phosphorus from 2.41 to 3.01 mg. Phosphorus supplements fed in the form of cottonseed cake, steamed bone meal, and mill-run bran increased the blood inorganic phosphorus to near that reported as optimum by some workers or 5 mg per 100 cc of blood serum. It was lowest in the lot receiving ground barley and highest where steamed bone meal was fed. The various phosphorus supplements produced little, if any, effect upon the blood calcium. A low negative

correlation was found between the inorganic phosphorus and calcium of the blood. There was a close correlation between the phosphorus intake and the inorganic phosphorus of the blood. The question is raised: Is it possible that many cattle (even in Utah where the soil in most cases is rich in phosphorus) may lack phosphorus in their ration? It appears feasible that this could be determined by blood analyses.

LITERATURE CITED

(1) Armsby, H. B.
1917. The nutrition of farm animals. 743 pp., illus. New York.

(2) Becker, R. B., Eckles, C. H., and Palmer, L. S.
1927. Effect of mineral deficiency on the yield and composition of cow's milk. Jour. Dairy Sci. 10: 169–175.

(3) Du Toit, P. J., Malan, A. I., and Groenewald, J. W.
1931. Studies in mineral metabolism. XVII. Phosphorus in the nutrition of sheep (2nd report). Union So. Africa Dept. Agr. Rept. Dir. Vet. Research 17: 453–472, illus.

(4) Eckles, C. H., Becker, R. B., and Palmer, L. S.
1926. A mineral deficiency in the rations of cattle. Minn. Agr. Expt. Sta. Bull. 226, 49 pp., illus.

(5) ——— Gullickson, T. W., and Palmer, L. S.
1932. Phosphorus deficiency in the rations of cattle. Minn. Agr. Expt. Sta. Tech. Bull. 91, 118 pp., illus.

(6) Forbes, E. B., Schulz, J. A., Hunt, C. H., Winter, A. R., and Remler, R. F.
1922. The mineral metabolism of the milch cow. Jour. Biol. Chem. 52: 281–315.

(7) Green, H. H., and Macaskill, E. H.
1928. Studies in mineral metabolism. VI. Comparison of blood of cow and calf in respect to mineral constituents. Jour. Agr. Sci. [England] 18: [384]–390.

(8) Harnes, A. R.
1928. Biometry of calcium, inorganic phosphorus, cholesterol, and lipoid phosphorus in the blood of rabbits: I. Normal animals from recently acquired stock. Jour. Expt. Med. 48: 549–565, illus.

(9) ———
1929. Biometry of calcium, inorganic phosphorus, cholesterol, and lipoid phosphorus in the blood of rabbits: II. Repeated observations on normal animals. Jour. Expt. Med. 49: 287–301, illus.

(10) Hart, E. B., Steenbock, H., and Humphrey, G. C.
1920. Influence of rations restricted to the oat plant on reproduction in cattle. Wis. Agr. Expt. Sta. Research Bull. 49, 22 pp., illus.

(11) Hawk, P. B., and Bergeim, O.
1931. Practical physiological chemistry; a book designed for use in courses in practical physiological chemistry in schools of medicine and science. Ed. 10, rewritten and reset, 929 pp., illus. Philadelphia.

(12) Huffman, C. F., Robinson, C. S., and Winter, O. B.
1930. The calcium and phosphorus metabolism of heavily milking cows. Jour. Dairy Sci. 13: 432–448.

(13) Kellner, O. [J.]
1907. Die ernahrung der landwirtschaftlichen nutztiere. Lehrbuch auf der grundlage physiologischer forschung und praktischer erfahrung. Ed. 4. Berlin.

(14) Malan, A. I.
1930. Studies in mineral metabolism. XI. Mineral metabolism and blood analysis. Union So. Africa Dept. Agr. Rept. Dir. Vet. Research 16: 307–311.

(15) ——— Green, H. H., and Du Toit, P. J.
1928. Studies in mineral metabolism. V. Composition of bovine blood on phosphorus deficient pastures. Jour. Agr. Sci. [England] 18: [376]–383.

(16) MEIGS, E. B., BLATHERWICK, N. R., and CARY, C. A.
1919. CONTRIBUTIONS TO THE PHYSIOLOGY OF PHOSPHORUS AND CALCIUM METABOLISM AS RELATED TO MILK SECRETION. Jour. Biol. Chem. 37: 1–75.
(17) ——— BLATHERWICK, N. R., and CARY, C. A., with the collaboration of WOODWARD, T. E.
1919. FURTHER CONTRIBUTIONS TO THE PHYSIOLOGY OF PHOSPHORUS AND CALCIUM METABOLISM OF DAIRY COWS. Jour. Biol. Chem. 40: 469–500.
(18) ——— and TURNER, H., with the cooperation of HARDING, T. S., HARTMAN, A. M., and GRANT, F. M.
1926. CALCIUM AND PHOSPHORUS METABOLISM IN DAIRY COWS. Jour. Agr. Research 32: 833–860, illus.
(19) PALMER, L. S., CUNNINGHAM, W. S., and ECKLES, C. H.
1930. NORMAL VARIATIONS IN THE INORGANIC PHOSPHORUS OF THE BLOOD OF DAIRY CATTLE. Jour. Dairy Sci. 13: 174–195, illus.
(20) ——— and ECKLES, C. H.
1927. EFFECT OF PHOSPHORUS DEFICIENT RATIONS ON BLOOD COMPOSITION IN CATTLE. Soc. Expt. Biol. and Med. Proc. 24: 307–309.
(21) ——— GORTNER, R. A., and RUDE, R.
1930. THE BIOMETRY OF CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD PLASMA OF DAIRY CATTLE. APPLICATION OF RESULTS TO BONE MINERALIZATION. Jour. Dairy Sci. 13: 360–367.
(22) PEARL, R.
1923. INTRODUCTION TO MEDICAL BIOMETRY AND STATISTICS. 379 pp., illus. Philadelphia.
(23) PETERS, J. P., and VANSLYKE, D. D.
1931–32. QUANTITATIVE CLINICAL CHEMISTRY. 2 v., illus. Baltimore.
(24) ROBINSON, E. M.
1929. NOTES ON BOTULISM IN THE DOMESTICATED ANIMALS. Union So. Africa Dept. Agr. Rept. Dir. Vet. Research 15: 97–110.
(25) THEILER, A., and GREEN, H. H.
1932. APHOSPHOROSIS IN RUMINANTS. Nutrition Abs. and Rev. 1: 359–385.
(26) ——— GREEN, H. H., and DU TOIT, P. J.
1924. PHOSPHORUS IN THE LIVE STOCK INDUSTRY. Jour. Dept. Agr. So. Africa 8: 460–504, illus.
(27) ——— GREEN, H. H., and DU TOIT, P. J.
1928. MINIMUM MINERAL REQUIREMENTS IN CATTLE. Jour. Agr. Sci. [England] 17: 291–314, illus.

SURVIVAL OF BLISTER-RUST MYCELIUM IN WESTERN WHITE PINE [1]

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INTRODUCTION

Studies on the growth and behavior of cankers of white pine blister rust (*Cronartium ribicola* Fischer) on western white pine (*Pinus monticola* Dougl.) have been reported by the senior writer in a previous paper.[3] That paper contains data on the manner in which canker growth rates are influenced by the size and vigor of the infected stem, by regional site conditions, and by girdling and subsequent "flag formation"—a term descriptive of the conspicuous red-brown color of the foliage of the affected part after death. In that paper (p. 594) reference was made to a study of the survival of the mycelium in trunk cankers; the results of that study are presented herein.

PROCEDURE

During 1922 and 1923 the senior writer observed that blister-rust cankers on branches of western white pine in British Columbia continued to grow toward the bole of the tree after the outer portion of the branches had died and turned into "flags", and in some cases after squirrels had girdled or severed the branch at the center of the canker.[4] In order to verify the results of these observations and to determine the extent to which the rust could continue to live and grow after all source of food supply had been cut off beyond the point of infection, a number of cankered branches were so cut that part of the infected bark remained on the stub and these stubs were examined at fixed intervals. On April 25, 1924, 34 infected branches were selected on thrifty western white pines at Chee Kye,[5] British Columbia, their diameters at the base of the canker were measured, and they were then lopped off just above the lower limits of the typical orange discoloration which closely follows the progress of the mycelium in the bark of western white pine. Throughout the remainder of this paper all cankers shortened in this manner are referred to either as "cut" cankers or as "truncated" cankers. On October 25, or 6 months from the time of cutting, all the cankers were in good condition and had continued to extend toward the bole. The downward growth of the mycelium, as indicated by the downward extension of the discoloration of the infected bark, was carefully measured for each canker.

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[2] The writers acknowledge indebtedness to C. N. Partington, J. L. Mielke, and T. S. Buchanan for assistance in carrying on the field studies upon which this article is based.
[3] LACHMUND, H. G. GROWTH AND INJURIOUS EFFECTS OF CRONARTIUM RIBICOLA CANKERS ON PINUS MONTICOLA. Jour. Agr. Research 48: 475-503, illus. 1934.
[4] For several years numerous investigators have observed that squirrels and other rodents frequently eat the infected bark of white pine blister-rust cankers. Occasionally they completely sever the branch at the point of infection.
[5] Chee Kye is a stop on the Pacific Great Eastern Railway, approximately 40 miles, air line, north of Vancouver, British Columbia.

In April 1925 a series of 50 uncankered thrifty branches on the same area were cut in the same way that the cankers were cut the preceding year. These checks were established to determine how long uninfected branches would remain alive after cutting. In July, 42 were dead and the remaining 8 died within 2 months. During this time most of the stubs of the cankered branches cut in 1924 remained alive and the mycelium continued its growth from their cut ends toward the boles. This proved beyond a doubt that it was the presence of the mycelium in the cut cankered stubs which had kept them alive.

In the latter part of September 1925 a forest fire swept over the area and terminated the entire study before the 34 cut cankers were remeasured. Therefore growth measurements were secured for a 6-month period only—from April 25 to October 25, 1924. A comparison of the growth rate of these cut cankers with that of normal uncut cankers in the same area indicated that the downward growth rate of the cut cankers was somewhat less in each diameter class than it was for the corresponding uncut cankers. The data were too few to be conclusive; therefore plans were made for a more complete study.

A western white pine infection area near Owl Creek,[6] British Columbia, was chosen for the second experiment. Pine infection first occurred there in 1917, increased considerably in 1921, and became common in 1923 and 1924. In 1927 at the beginning of the test the white pines were young and still thrifty. They were approximately 12 to 25 years old and from 9 to 22 feet tall.

On May 6, 1927, 100 cankers on thrifty branches of 32 trees were carefully selected. All these cankers were of 1923 or 1924 origin and were therefore relatively young, most of them having produced aecia only once or twice. In general, they were one internode distant from the bole; a few, however, were almost two internodes distant. The distance from the bole to the lower limits of the cankers ranged from 2 to 17.3 inches, being more than 8 inches in only a small number of cases. The diameter of the branches at the lower limits of the cankers ranged from 0.2 to 0.9 inch. For each 0.1-inch class between these limits the number of branches was approximately the same.

Each branch was severed by a cut perpendicular to its long axis at a point about one half of an inch above the lower limit of the discoloration. On the same trees, 100 uninfected branches were cut in identical fashion to serve as checks. The check for each cut canker was on a branch of the same size and corresponding location in the tree.

In no case were any living needles or small side branches left between the cut surface and the bole; therefore it was impossible for any of the cankered stubs or checks to obtain food except from the main stem, or bole.

These truncated cankers and checks were examined in the fall of 1927 and in the spring and fall of each successive year until the spring of 1932. The condition of each was noted—whether living or dead—and, in the case of the living cankers, the downward growth from the lower limit at the time of cutting or at the time of the preceding examination was accurately measured. Of the original 100 cankers,

[6] Owl Creek is a stop on the Pacific Great Eastern Railway, approximately 35 miles north of Chee Kye.

22 were discarded before the completion of the study because of the death of the trees on which they were located.

On May 1, 1928, a similar experiment was begun on the same area but on different trees. Fifty cankered branches and fifty uninfected checks were cut in exactly the same way as were those in the preceding year. In order to determine the immediate effect of cutting, this series was examined monthly until fall; after that the data were taken as for the 1927 series. Here again the basis for comparisons was reduced through the death of some of the trees. Data were taken on 44 cut cankers and checks in 1929 and on 32 cankers and checks in 1930. At the end of 1930 work on this series was terminated.

SURVIVAL OF TRUNCATED CANKERS

The data summarizing the condition of the cankered stubs at half-yearly and yearly intervals are shown in table 1. The condition of the cankered stubs is assumed to be the same as that of the canker itself—i.e., living or dead—for *Cronartium ribicola* is an obligate parasite and can live only so long as its host remains alive. When a canker had grown down the stub and entered the bole, the stub died in a short time. Inasmuch as the purpose of this study was to determine the effect of cutting on canker survival and growth, those cankers that died or entered the bole were not examined at subsequent dates. Because of the uniformity of the data after the 2-year period, the half-yearly intervals are not shown.

TABLE 1.—*Survival of truncated cankers and checks, Owl Creek, British Columbia*

Cankers or checks (number)	Time after cutting	Cankers			Checks dead
		Living but not yet reaching bole	Dead before reaching bole	Entered bole	
	Years	*Percent*	*Percent*	*Percent*	*Percent*
122	½	95.9	0.0	4.1	95.9
122	1	91.0	1.6	7.4	99.2
122	1½	66.4	8.2	25.4	100.0
110 [a]	2	44.6	18.2	37.3	----------
78 [b]	3	21.8	21.8	56.4	----------
78	4	5.1	29.5	65.4	----------
78	5	3.8	30.8	65.4	----------

[a] Trees containing 12 cankers died during winter.
[b] Examination of 1928 series discontinued.

Examination of the data for the 122 checks shows that 95.9 percent, or 117, were completely dead half a year after they were cut. Only 1 of the remaining 5 checks survived for 1 year, and it died shortly thereafter.

Comparison of the survival data for the truncated cankers with those for the checks clearly shows that the former remained alive for a very much longer time than the latter. This comparison is further strengthened by the results of the monthly examination of the checks in the 1928 series. Five percent of them were dead 3 months after cutting and 90 percent in 4 months, showing that the great majority of deaths occurred during the first 6 months.

In a previous reference to the present study the statement was made [7] that the survival of the cankered stubs suggested that "the presence of the infection at the end of the stub had the effect of stimulating a reversal of flow of the elaborated food materials." The data presented herein are believed to justify fully this hypothesis. The fact that with one exception the uninfected checks died within a year after cutting whereas the cankered stubs remained alive in some cases for at least 5 years shows that the presence of blister-rust infection tended to keep the stubs alive until the cankers could grow into the bole.

The action of the rust upon the stream of assimilates in the cut cankered stubs would be an interesting subject for physiological study. No other phenomena even remotely analogous to the survival of the cankered stubs are known to the writers. Certain features of resemblance are suggested by the well-known action of false-mistletoe infection, which has the effect on several hosts of prolonging the life of certain branches; notably in the case of the lower branches of *Pinus ponderosa* Dougl. and *P. contorta* Dougl. But in these cases the prolongation is attributable to a stimulation of the growth of the foliage beyond the point of infection whereby the infected portions are provided with an increased supply of elaborated food from the normal direction.

DOWNWARD GROWTH OF TRUNCATED CANKERS

As previously stated, the downward growth of all living cut cankers was carefully measured at the time of each examination. These measurements, too numerous for detailed publication, indicate three things: (1) The downward growth rate of the mycelium in the cankered stubs was constant as long as the stubs remained alive; (2) this rate was very slightly less than the downward growth rate of normal uncut and unflagged cankers; and (3) it was almost identical with the downward growth rate of flagged cankers, which is likewise slightly less than the normal growth rate. This reduced rate of growth can obtain, however, only while the fungus is in the stub or in those portions of the stem below the flag which are in low vigor because of the flagging. As soon as the infection reaches the bole or the region of the healthy living side branches below the canker, its rate of growth immediately becomes normal.

While similar in rate of downward growth, the cut and the flagged cankers are not exactly comparable as regards the length of time they may remain alive on internodes unsupported by living foliage beyond the canker. The death of the portion of the branch or stem beyond the canker in flagging reacts differently and evidently saps the vitality of the lower uninfected portion much more than when the branch or stem beyond the canker is cut off. For example, when a canker is situated so far down on a branch that most of the foliage is beyond the point of infection, the entire branch is generally killed at the time of flag formation. It has been noted, further, that flagged cankers almost invariably die out if the length of unsupported internode is so long that the canker cannot grow over it within 2 years. On the other hand, the present data show that the majority of the cut cankers

[7] LACHMUND, H. G. See footnote 3.

remain alive for at least 2 years and that many of them survive for 3 years or more. From these observations it appears that cutting causes less of a drain on the lower stem than does flagging. Consequently the longevity and survival data for the cut cankers cannot be used as criteria for the survival of cankers after flagging.

SUMMARY

The mycelium of white pine blister rust continues to live and grow in infected western white pine bark after flagging, i.e., the death of the portion of the branch beyond the canker, has taken place or after squirrels have girdled or severed the branch at the center of the canker. In order to obtain definite information on this phenomenon the writers in the spring of 1927 and 1928 shortened 150 cankers, leaving stubs with about one half of an inch of living canker at their tips. Checks were cut in exactly the same way on uninfected branches in the same relative position in the trees.

The checks were practically all dead within 1 year from the date of cutting, whereas the cankered stubs remained alive for periods ranging up to 5 years, or until the mycelium entered the bole of the tree. The mycelium apparently stimulated a reversal of the flow of assimilates in the infected stubs.

Measurements of the downward growth of the cankers in the stub indicate that it proceeds at a constant rate slightly less than that of normal cankers but almost identical with that of flagged cankers. Flagged cankers survive for a shorter time than cut cankers.

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